SUBJECT OF THESIS.

TO DETERMINE THE INCIDENCE OF

BRUCELLA ABORTUS

AND CERTAIN OTHER ORGANISMS IN TONSILS.

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CONTENTS.

GENERAL INTRODUCTION

I. Main object of the Investigation

The primary object of the investigation recorded in this thesis was to determine, if possible, the frequency in which the Bacillus abortus of Bang could be recovered from tonsil tissue.

It is obvious that normal tonsils would not be available for this study and, therefore, one was compelled to use organs which had been removed for one reason or another - "enlarged tonsils". From the stand point of the investigation, however, these might be regarded as normal tonsils in this sense that none of the patients from whom they were derived showed any evidence of Undulant Fever, whose causal agent - the Bacillus abortus - was specifically sought.

While it has been reported by several American observers (Mohler and Traum, 1913., Poelma and Dickens, 1932, and Carpenter and Boak, 1932) that B. abortus can sometimes be isolated from tonsils, we are not aware of any similar investigation having been carried out in this country.

It might be argued that this study was of purely academic interest, but in a statistic of 850 samples of fresh milk in the City of Dundee no less

than 18% were found to be infected with the B. abortus, (Morton, 1933). It might well be then that the matter is not without practical significance.

The position is that roughly every fifth glass of raw milk is infected, and it is pertinent to ask what might become of the micro-organism when such milk is consumed.

There are 3 possibilities

(1) That this organism rapidly passes through the gastro-intestinal tract making no lodgment therein.

(11) That it does lodge in the tract for some time, but being devoid of high invasive properties, so far as human tissue is concerned, behaves virtually as a saprophyte. It seemed possible that the tonsils might be a structure in which this might occur.

(111) That it lodges somewhere, e.g. in the tonsil and also <u>invades</u>. If this be so the invasion can only be circumscribed, (in most cases at least), for the occurrence of cases of declared Undulant Fever in the human due to the B. abortus is very infrequent when the volume of infected material which is consumed is borne in mind.

The interest in the matter is not, however, limited to these considerations for there are two outstanding points that merit enquiry.

In the first place it is probable that an appreciable number of people have acquired infection with the Bacillus abortus unconsciously, and thus out of 1,943 specimens of human sera, submitted for the Wassermann Test, 6 agglutinated B. abortus in a dilution of 1/200 or more, 5 reacted in a dilution of 1/100 but not 1/200, 5 reacted to a dilution of 1/50, and 23 reacted in a dilution of 1/25. (Communicated by Prof. Tulloch, Dundee). Expressed as a percentage the figure is not high, but on the other hand it is scarcely negligible. It is possible that such people have really been cases of a marked degree of latent infection, and the site of such latent infection could be tonsillar tissue.

Secondly, it has been so far assumed that the immunity to abortus infection exhibited by the genus homo is an innate natural immunity; it is possible, however, that it really depends on the acquisition of a natural active immunity, due to exposure to "sub-clinical infections", and, therefore, comparable to the natural acquired immunity of the average adult, for example, to Diphtheria.

If this second hypothesis be correct, then it is improbable that infective material passes through the gastro-intestinal tract without lodgment of some kind, and therefore it is of some

importance to determine whether the tonsil is a common site of "sub-clinical infections" with the micro-organism in question. In order to be assured that a milk-consuming section of the community was chosen for investigation the source of the material was limited as far as possible to tonsils obtained from children under five years of age. Even with this precaution, however, one cannot assume that these children all consumed raw milk, for a large proportion of the milk consumed in Dundee is pasteurised and the housewife may purchase the commodity without knowledge that it has been thus treated, prior to sale.

For this reason alone one must expect to demonstrate the organism only in a relatively small percentage of the cases investigated. Moreover, we do not know the duration of the minimal infection of Bacillus abortus and it is, therefore, possible that many specimens which may have been infected at one time, do not happen to be infected when the tissue was removed.

For these reasons then the demonstration that even a small number of tonsils harbour the B. abortus would not be without significance.

II. Subsidiary Objects of the Investigation.

(a) A Possible Fallacy in the Inoculation Method for Demonstrating the Presence of B. abortus in Morbid Material.

The method usually employed for demonstrating the B. abortus of Bang in morbid material is to inoculate that material into a guineapig, which is killed after 4 to 8 weeks, when cultures are made from the spleen pulp and an agglutination test performed with its serum.

This method assumes that the guineapig is a delicate indicator of abortus infection: (as reported by Hagan, (1922) who worked with pure cultures). One may doubt whether this assumption is valid when working with Tonsil tissue, which contains many species of organisms.

It is a suggestive fact that the guineapig can harbour Bacillus abortus in its internal organs without apparently showing any ill effect, and this may indicate that the guineapig will become infected only when an overwhelming dose of the organism is introduced into its tissues. In other words, it may well be that the threshold dose required to infect the guineapig is high, and if that be so, then all statistics based on the biological (guineapig inoculation) test would be unreliable.

For this reason it was decided to attempt to elaborate culture methods which might in themselves be used to isolate the micro-organism in question, or alternatively, might be employed to enrich the material wherewith to conduct the

biological test.

It was realised that this must present very considerable technical difficulties, because of the rich and varied flora of the tonsil. Nevertheless cultural isolation was worthy of extended trial, not because it was felt that cultural investigation would be of great value for the specific purpose of demonstrating Bacillus abortus infection, but because any information gained might be of use as indicating procedures applicable to the isolation of that micro-organism from bovine milk. If even a relatively satisfactory cultural method of isolating B. abortus from milk could be elaborated, quite extensive milk surveys might be made economically.

(b) Investigation of Other Species of the Tonsillar Flora.

As the material was available it was decided that a superficial study at least of some strains of the tonsillar flora might with advantage be carried out, while the specific investigation concerning B. abortus was proceeding.

Even a superficial study of all the constant micro-organisms of the tonsillar flora would be a subject of magnitude and, therefore, this survey was limited to the investigation of :-

1. Small Gram-negative bacilli of the tonsil. (Influenza group bacilli).
2. Streptococci. (Haemolytic Strains).
3. Any concomitant infection encountered, although not specifically sought. e.g.

B. tuberculosis in the inoculated guineapigs.

1. Gram-negative Bacilli of the Tonsil.

All the tiny Gram-negative bacilli of tonsillar origin that might be encountered in this study were of interest because of their morphological resemblance to Bacillus abortus. The majority of such organisms might be expected to belong to the haemophilus group of bacteria, and any which might prove to be outwith that group would be specially interesting because of their resemblance to B. abortus, and because of the difficulties to which their presence would give rise in attempting cultural isolation of that bacillus.

Apart from this specific interest, the Gram-negative bacilli of the tonsil are of general interest, in that Fleming and MacLean, (1930), have recently claimed to be able to demonstrate the presence of haemophilic bacilli in the upper respiratory tract of approximately 100% of humans. It is obviously of interest to verify this finding and, if possible, to amplify it, using the highly specialised technique of these workers.

To this end we thought it would be of value to submit all strains to the various tests recommended by different observers (oxidase reaction, tellurite tolerance, mouse pathogenicity etc.,), in

order to discover whether any correlation exists between these in the group of haemophilic bacilli. It would be essential to first classify them according to their growth requirements (X & V factors), as dependence upon one or both of these factors is the outstanding characteristic of this group of organisms.

This proved to be an extensive investigation and was carried out as thoroughly as possible.

11. Streptococci.

Owing to the constant presence of Streptococci of one kind or another in the tonsillar tissue it was decided to restrict this phase of the investigation to a superficial survey of the prevalence of haemolytic streptococci in the material handled.

111. Concomitant organsims encountered though not specifically sought.

These included the following:-

1. Bacillus tuberculosis

The guineapigs used in our search for B. abortus would also indicate the presence of B. tuberculosis especially if kept for 6 or preferably 8 weeks after inoculation as demonstrated by Morton(1933). It would be of great interest to know the incidence of this organism in childrens'

tonsils, bearing in mind that pasteurisation might render the positive findings very small as in the case of B. abortus.

11. Micrococcus flavus.

As the work proceeded it became plain that Gram-negative cocci producing yellow pigment, and giving homogeneous suspensions in saline were very frequently encountered in the cultures (when isolating the Influenzoids). During part of the investigation their presence was noted and they are referred to herein by the designation "Flavoid" cocci. They form part of the basic flora of the throat and tonsils and appear to be harmless.

111. The only other organismsencountered which seemed worthy of note were B. welchii and B. diphtheriae,

We have divided this thesis accordingly into 4 Sections corresponding to the organisms investigated as follows.

Section I. B. abortus.

" II. B. influenzae (group)

" III. Haemolytic Streptococci.

" IV. Other organisms (a) B.tuberculosis
(b) M.flavus
(c) B. welchii
and B. diphtheriae

Each Section is divided into 2 parts -

Part I contains a review of the literature dealing with the particular organism, and Part II consists of the present investigation of each organism or group.

Results are summarised in each Section and a list of references appended.

SECTION 1.

To determine the incidence of

Brucella Abortus in Extirpated Tonsils.

PART I.

Review of the Literature dealing with Bacillus abortus Infection in man.

The question of the infectivity of B. abortus for man has received increasing attention during the past 10 to 15 years. This is reflected in the enormous literature which has accumulated on this subject during that period. Whilst our laboratory investigation was confined within definite limits (namely, to search for B. abortus in tonsils), it seems desirable to make a general survey of the most important contributions to the literature dealing with the whole subject. This should serve as a background for our study.

The literature bearing on this subject is somewhat complex involving 2 main currents, medical and veterinary, but also various side streams, such as examination of cow's milk on the one hand and the blood serums of normal human beings on the other hand, besides the more direct investigation of the condition known as Undulant Fever. It would seem best to review the development of our knowledge as far as possible in its historical order.

As the Micrococcus melitensis of Bruce, the causal organism of Mediterranean or Undulant Fever in man, has been shown to be very closely related to B. abortus of Bang, (both now being classed together

under the generic name Brucella), it will be necessary in our survey to make frequent reference to the former organism and its effects.

Up to the time of the Crimean War various fever conditions occurring in the Mediterranean were known under the names of Continued Fever, Remittent Fever, or Continued Remittent Fever. Amid this chaos Marston identified one type as a clinical entity, and called it Mediterranean or Malta Fever. He gave the first full and accurate description of it in 1861. Twenty six years later the causal organism was isolated from the spleen of a fatal case, by Bruce (1887) in Malta and called the Micrococcus melitensis. It was a tiny Gram-negative coccobacillus.

Dudley (1931), gives an interesting account of the history of Mediterranean (or Malta) Fever in the British Navy. This condition was first given a separate heading in naval returns in 1897. He gives statistics which show how very prevalent this fever was amongst the personnel of the fleet stationed at Malta prior to 1905. A few hundred cases occurred annually, and although the death rate was only about 2% or 3%, it caused much invalidism, many being unfit for further service.

The fullest description of the fever is given by Hughes in his monograph. The fever is

prolonged, the average duration being 3 months, and it is characterised by anorexia, headache, insomnia, sweating, rigors, waves of pyrexia, loss of weight, joint pains, anaemia, leucopenia, enlarged spleen, general asthenia, and tardy convalescence. Some cases go on for 1 or 2 years. Complications include arthritis; endocarditis; orchitis; and bronchitis; general asthenia being the most common sequela. No treatment has any appreciable influence upon the course of the disease, although occasionally the use of vaccine or chemotherapy seems to be helpful.

It was evident that this fever was a very serious menace, not only to the fleet, but also to the military personnel, as well as to the civilian population in Malta. Accordingly a Royal Commission was appointed, with Colonel (afterwards Sir David) Bruce as chairman, and they investigated the disease in Malta from 1904 to 1906. In this island goats are the chief source of the milk supply, and the Commission proved that these animals formed the chief reservoir of the specific organism, the Micrococcus melitensis. It was harboured in their mammary glands and udders and discharged in their milk. The goats may appear quite healthy and yet give infected milk. The number of infected goats varies in different herds - but averaged 10 to 20%. It has since been established by Zammit (one of the Commission) that once a goat is infected, it remains

a permanent carrier.

The application of the Commission's findings had dramatic results. Goats' milk, which was widely used by both Army and Navy, was either prohibited or had to be boiled before use - resulting in an almost total disappearance of the disease amongst the personnel of these services.

The Royal Commission also confirmed the use of the serum agglutination test (first demonstrated by Wright in 1897) as a means of diagnosing the infection, both in human beings and in goats. They also found that agglutinins to the M. melitensis were present in the milk of infected goats. Following this, cases of this fever were discovered in many parts of the world, besides the Mediterranean littoral, including places in Africa and India, North and South America, West Indies, etc. Geographical designations for the disease were seen to be inadequate and misleading, and the name Undulant Fever (first recommended by Hughes because of the characteristic waves of pyrexia) was generally adopted.

Meantime in the veterinary field a discovery had been made which was to be shown later to be closely linked with those which we have discussed. In the year 1897 Bang in Denmark discovered the causal organism of contagious abortion of cattle - an epizootic disease of world wide

distribution. It was a tiny Gram-negative bacillus, or coccobacillus, and he called it Bacillus abortus. This organism was not isolated in England till 1909 and in U.S.A. till 1911, (according to Cooledge 1916)

Schroeder & Cotton (U.S.A.) in 1911 were the first to isolate B. abortus from cow's milk. They concluded that "(1) the bacillus of infectious abortion may and in most cases does persist, in the udders of cows that have aborted, for years and possibly for the balance of their lives; and during this time is eliminated more or less continuously in their milk. (2) It may be eliminated for years from the udders of cows that have never aborted."

They found 10% to 14% of the cows' milk samples in Washington which they inoculated into guineapigs contained B. abortus . McFadyan and T. Smith in 1912 made similar observations which were confirmed by Zwick and Krage in 1913 and Fleischner and Meyer in 1917.

In 1913 Larson and Sedgwick examined the serums of 425 children by the Complement Fixation Test and found 17% positive to B. abortus, whilst Ramsay in 1915 making a similar examination in children found 6% positive.

In 1914 Kennedy showed that the milk and serums of some London cows possessed the power of agglutinating cultures of M. melitensis. This was

confirmed by Bassett-Smith in the same year, Kennedy adds " I have heard of 2 cases of Undulant Fever in people who had never been out of England and it is possible thatthere are others undiagnosed". The significance of these observations was not appreciated till later.

Following these discoveries Nicol and Pratt (1915) and Cooledge (1916) made enquiries into the possibility of B. abortus being infective for man, but they apparently did not come to any definite conclusions.

The close relationship between M. melitensis and B. abortus had not yet been discovered. This illuminating discovery was made by Miss Alice C. Evans (U.S.) in 1918. She showed that the M. melitensis and B. abortus bore so close a resemblance as to be almost identical. They could not be distinguished from each other by their morphological or cultural characters, nor by ordinary agglutination reactions. But she showed that they could be differentiated by the <u>agglutinin absorption test</u>; and by this means she classified 68 strains into at least 8 serological groups.

The 2 important discoveries she made were (1) the <u>very close relationship</u> between M. melitensis and B. abortus - so close that some regard them as varieties of the same species. (Brucella).

(2) the value of the agglutinin absorption method for differentiating between members of this Brucella group.

These findings of Evans were corroborated by Khaled (1921), Burnet (1922) & Meyer & Shaw (1920).

These results opened up a very interesting and expansive field of speculation and research, and at the same time threw light upon the findings of previous investigators, such as those of Larson and Sedgwick, and Kennedy. The suspicion that B. abortus might be pathogenic for man was strengthened by Evans' discovery, and research was stimulated along those lines. Observations were made by both medical men and veterinarians in the United States, in South Africa and in Europe, a very large proportion of the work being done on the other side of the Atlantic. Evidence gradually accumulated which proved the B. abortus to be infective for the human organism. We shall as briefly as possible survey the growth of this evidence.

We may note here that the terminology of these organisms was modified in accordance with Evans' findings. Both organisms were classed under the generic name Brucella (after Bruce), the specific names melitensis and abortus being retained for the caprine and bovine strains respectively

The first evidence is that supplied by

Bevan (1921-22) in South Africa. It must be admitted that it is circumstantial only, although very strongly suggestive. Among his conclusions he says, "Certain cases presenting symptoms resembling Undulant Fever have occurred in Southern Rhodesia in men, who as far as is known have not obtained infection from goats, but have resided on farms where animals are or have been infected with infectious abortion. It is suggested that these cases are suffering from an infection caused by B. abortus of cattle." and he further states, "While circumstantial evidence points to the infection of man by the B. abortus of Bang and scientific tests up to a point support this suspicion, the Final proof is not yet available." Bevan was apparently the first to call attention to the possible danger to man arising from infection of dairy products from a herd infected with contagious abortion.

The first case in which the "final proof" was supplied, was that reported by Keefer in Baltimore in 1924. He isolated an organism from the blood and urine of his patient and by agglutinin absorption tests identified it as Bacillus abortus (Bang). The patient, a youth of 19 had symptoms resembling "Malta Fever", he drank a good quantity of cow's milk and ate a good deal of cheese. Keefer says, "This is the first recorded case of a disease in man corresponding to Malta Fever due to an organism belonging to the Abortus group."

In the same year (1924), Orpen, shortly after Keefer, isolated an organism serologically indistinguishable from B. abortus from the blood of patients suffering from Undulant Fever in Southern Rhodesia. Duncan in the same year made a similar finding (in England) in an Undulant Fever patient who had come from that colony. The organism he isolated corresponded more closely to B. abortus than to M. melitensis. By 1925 Bevan reported 35 such cases had occurred in Southern Rhodesia.

Ficai and Alessandrini (1925) and Viviani (1925) record cases of Undulant Fever due to contact with aborting cows, and presumed to be due to B. abortus. But as agglutinin absorption tests were not done, and as B. melitensis infection may occur in cows in Italy - these reports are inconclusive.

In 1926 Huddleson published similar results regarding 3 cases, Carpenter 5 cases, and Carpenter and Merrian 2 cases. In Germany Kreuter (1926) and Spreugel (1926) each reported a case - both in veterinaries. (Poppe 1929).

Regarding mode of infection this appeared to vary in different cases. Carpenter and Merrian for example, state that, "the only source of infection known in their cases was milk - Both patients were in the habit of ingesting comparatively large amounts of milk daily. The milk consumed by one of them was examined and found heavily infected with B. abortus.

Goats and swine as sources could be excluded." In 2 of Huddleson's cases, on the other hand, infection was almost certainly due to handling of living cultures of B. abortus, as they were laboratory workers.

In the German cases contact with infected animals seemed to be the cause.

We shall see that this "contact infection" plays an important part in the incidence of human abortus infection or "abortus fever", as it is frequently termed. Thus amongst veterinarians, farm workers, slaughtermen and packing house workers it has been shown to be practically an occupational disease. The large majority of such cases occur in males, (who form the majority of such workers). Where milk is the source of infection, however, males and females are almost equally infected.

We may note here that other domestic animals besides cattle have been found infected with B. abortus. These include swine (porcine strain), sheep, horses, and occasionally dogs and cats. Fowls may also become infected from cattle and pigs; the organism settles in the ovaries and may be found in the eggs; making fowls possible sources of infection at farms. (Mackie, 1933). Swine are a frequent source of human infection in the United States; whilst in France sheep are a more common source than cows.

The sheep are apparently usually infected with Br. melitensis (from goats).

The essential similarity between the Br. melitensis infection in goats and the Br. abortus disease in cattle&swine has also been elucidated. The typical disease in all these species (as well as in others) is seen in the pregnant females, the chief symptom being abortion, which usually occurs only once, but may occur oftener. Sterility sometimes results, and the disease causes great economic loss to farmers and others. It has been shown that goats not protected by inherited immunity, suffer from abortion when first infected (or inoculated) with Br. melitensis.

Evans (1923) inoculated a pregnant heifer (intravenously) with Br. melitensis and abortion followed. The organism was recovered from foetus and colostrum.

Once infected, a varying percentage of animals tend to remain so indefinitely, the organism being discharged in their milk. Sheather (1926) gives 30%, Klimmer and Haupt (1923) 41%, and Kern (1928) 60% as proportion of infected animals which give infected milk. There are usually no obvious lesions of the mammary glands or udders, and the animals appear healthy.

The most potent source of infection appears

to be by contact with the infected foetus and placenta, the genital passages, and uterine discharge following parturition (in those attending the animals). The uterine infection usually clears up after a week or two, but those animals in which the milk has meantime become infected tend to remain permanent carriers.

We may regard the period 1918 to 1926 as that during which Br. abortus was under increasing suspicion of being pathogenic to man. From 1927 onwards the evidence from all quarters becomes more and more convincing. But conclusions regarding its pathogenicity did not go unchallenged.

Thus Nicolle, Burnet, and Conseil, in 1923 inoculated 5 humans with living cultures of Br. abortus (2 bovine, 3 procine strains) - with entirely negative results. They conclude "These results prove that the Br. abortus is not pathogenic for our species".

In 1924 Burnet did further inoculation experiments on 2 men and 2 monkeys. He concludes, "These experiments confirm that Br. abortus is not pathogenic for man or monkey".

In 1926 Theobald Smith reports, "In the meantime the evidence that Br, abortus producing disease of the placenta in cattle may produce a disease in man simulating Malta Fever must be

regarded as inadequate in establishing any such relationship".

The circumstantial line of argument was used by some, e.g. by Skaric in 1922. According to him contagious abortion is rife in some parts of Austria, yet there is no Undulant Fever to be observed in man in those same areas.

Bastai (in Italy)(1927) studied an outbreak of Undulant Fever around Turin - traced to cows - which in turn were infected from a flock of sheep with Br. melitensis.- He seems to regard all cases of human infection from cattle as due to Br. melitensis.

Some observers believed that where man has become infected from cows, the latter have been infected with Br. melitensis, not Br. abortus Bang. This criticism would apply more readily to areas like Italy and South France, where goats are frequently affected with melitensis, and these in turn infect the cows.

It will be seen however, that the most convincing evidence for the pathogenicity of Br. abortus comes from those countries where Undulant Fever had not been previously observed, and where contagious abortion of cattle is common - e.g., the Northern countries of Europe, (especially Denmark), and the northern United States. Infection

from goats in these parts can generally be easily excluded.

Several other observations prior to 1926 are worthy of note.

Mohler and Traum examined 56 Tonsils and Adenoids from milk-consuming children and found Br. abortus in 1 of them.(cited by Klimmer and Haupt 1922).

In Germany Klimmer and Haupt (1922) discuss the question, "Is the Br. abortus Bang pathogenic for man?" They refer to the occurrence of miscarriages amongst quite healthy women living in the country in which no evident cause was established. But enquiry showed that contagious abortion was prevalent amongst the cattle, and that the women concerned had been drinking raw milk. To consider that there is a connection between the epizoötic infection and the miscarriages, appears to them not unwarranted. On this point Carpenter (1926) quotes Larson and Sedgwick who observed that in Complement Fixation Tests of the serums from women who had aborted, the percentage containing abortus antibodies was greater than that giving positive Wassermann Reactions.

In 1917 Fleischner and Meyer as a result of guineapig inoculations with certified milk, concluded that Br. abortus for all practical purposes is

always present in the certified milk provided in the San Francisco Bay regions."

In 1924 Evans examined the blood sera of 500 patients suffering from various diseases and found that 59 (11.8%) contained specific agglutinins for Brucella.

Klimmer and Haupt (1923) examined the Dresden milk supply and found that 32% of the market milk samples contained Br. abortus.

Wilson and Nutt (1926) made similar investigations in Manchester (the first in this country), examining 488 milk samples. They found 5.7% single milks and 8.8% mixed milks contained Br. abortus.

From the figures quoted, and others available, it is evident that Br. abortus is consumed daily in large numbers by a large section of the population throughout many parts of the world - and as fresh investigations are set up in different countries it becomes more and more apparent that we are dealing with an almost world wide problem. This applies to both the veterinary and the medical aspects, for the two are inseparable. The whole question bears a parallel to that of tuberculous infection conveyed by cow's milk (bovine tuberculosis), but whereas children are mostly affected by this condition, and adults are comparatively immune, we shall see that the reverse holds good for

abortus infection.

From 1927 onwards the literature is very voluminous. A survey, as brief as possible, is given to illustrate the progress of our knowledge regarding this organism and the question of its pathogenicity for man. For the sake of clearness reports are grouped under the following headings, and include investigations regarding:-

1. Cultural characteristics of Br. abortus.
2. Abortus infection in cows (milk and blood serum).
3. Human serums from
   (a) normal individuals.
   (b) patients with undiagnosed fever (sent for Widal)
4. Abortus infection (Undulant Fever), in man.
   (a) in other countries.
   (b) in this country.
5. Br, abortus infection of reproductive organs (including human abortion).
6. Br, abortus infection in children.

1. Cultural characteristics of Br. abortus.

The Bacillus abortus of Bang (bovine) possessescertain cultural peculiarities which distinquish it from other organisms, including Br. melitensis.

From the first it was noted that freshly isolated strains would not grow under ordinary atmospheric conditions. It was found to grow, however, either (1) in sealed tubes or (2) when Bacillus Subtilis was grown in the same jar with it. (Method of Nowak), taking at least 3 or 4 days to grow.

It was supposed that reduced oxygen tension was the factor essential for growth of B. abortus. Stafseth (1920), reported good results using a jar partly exhausted of its air (oxygen).

By repeated subculture it was found that Br. abortus could become acclimatised to growing in ordinary air.

Huddleson (1921) advanced our knowledge of the growth requirements of this organism, when he pointed out that it grew in an atmosphere containing an increased Carbon dioxide tension, the optimum being about 10%.

This observation has been confirmed by many observers, e.g. T. Smith (1924) showed by experiments that concentrations of CO_2 from $\frac{1}{4}$% to 10% enabled Br. abortus to grow, and that even as

low a concentration as 0.1% favoured growth, although in a retarded manner. In all cases the resulting growth was very vigorous when compared with that in sealed tubes, and also more rapid, showing in 1or2 days.

In 1927 Huddleson, Hasley, and Torrey, explained the method of Nowak (growth along with Br. subtilis) and that of Preisz (sealed tube) as both being due to increased CO_2 tension and not reduced Oxygen tension. They also state that there is a similar increased tension of CO_2 in the fluids of the udder and pregnant uterus - averaging 10%.

These findings were confirmed by McAlpine and Slanetz (1928) who also noted that 5-10% CO_2 had a more or less inhibiting action on strains of porcine and human origin, and on Br. melitensis.

In this country the researches of Wilson (1931) have thrown further light upon this subject, showing that both oxygen and CO_2 are necessary for the development of Br. abortus (bovine), and that the optimal concentrations of these 2 gases are 20% and 5-10% respectively. He shows that a lowered oxygen tension is less favourable to growth than the pressure of oxygen in the air. He also concludes that the CO_2 acts, not by altering the acidity of the medium, but by increasing the intracellular Hion concentration.

It is now generally accepted that Br.abortus

(bovine strain) grows best in an atmosphere containing from 5-15% of CO_2. The procine and Rhodesian strains are, however, exceptions to this rule, as they grow well in ordinary air, as does also Br. melitensis.

Regarding suitable media Stafseth (1920) advocated liver and spleen media to aid isolation of this organism. Huddleson (1921) favoured liver infusion broth, and he (1927) refers to an animo acid, containing Sulphur, as forming the proper food for Br. abortus. It grows well however on Ordinary Agar and Fildes Agar, and still more luxuriantly on Boiled Blood Agar ("Chocolate agar"). We used this last named medium (modified) in our investigations.

This leads us to another characteristic of this micro-organism, namely, its power to produce Hydrogen Sulphide when grown on a medium containing Sulphur, as noted by Huddleson, Hasley, and Torrey, (1927).

Ammonia is also liberated in such a medium and combines with the magnesium phosphate (present in meat infusion) to form Ammonium Magnesium Phosphate, - which appears as visible crystals on the surface growth.

Neither of these phenomena occur with Br. melitensis, (or at least not to nearly the same extent) and so they serve to distinguish the 2 species

of Brucella.

Another means of differentiating between Br, abortus and Br. melitensis was by their reaction to certain dyes, first reported by Huddleson (1929). The dyes used were methyl violet, basic fuchsin, and thionin. Thus it was found that the bovine strain of Br. abortus grows on media containing either of the first 2 dyes, but not in presence of thionin, whilst the porcine strain is exactly the reverse of this. Br. melitensis grows well in presence of all 3 dyes. This bacteriostatic dye test has been applied by several observers in classifying strains they had isolated, e.g. Plastridge and McAlpine (1930) and Kristensen (1931) (along with H_2S test) and Taylor, Lisbonne, and Roman (1932) (who classify 191 French Strains.)

A further method of distinguishing between bovine and porcine strains of Br. abortus is that introduced by McAlpine and Slanetz (1928). They observed that porcine strains utilise appreciable amounts of dextrose (when added to the medium), whilst bovine strains use little or none. Plastridge and McAlpine (1930) used this method along with the dye test to differentiate 129 Strains of Brucella isolated from cases of Undulant Fever in United States and Europe, They found 63 were bovine type, and 66 porcine. Br. melitensis apparently utilizes dextrose well, similar to the porcine strain.

Our most important consideration, however, from the practical point of view, is, how can we isolate Br. abortus from infected material, e.g. animal tissue and fluids, such as milk, tonsils, etc?

To this end 2 methods have been utilized:-

(1) Animal inoculation.
(2) Culture method.

(1) Animal Inoculation.

The guineapig has been found the most useful laboratory animal for this purpose, being very susceptible to infection with Br, abortus.

As early as 1893 Smith and Schroeder noticed a disease in guineapigs following injection of milk, but the nature of it remained obscure. In 1911 Schroeder and Cotton noticed the similarity of this disease with that caused by experimental inoculation of Br. abortus; and on making cultures from the affected guineapigs they were able to prove their identity. Since then the presence of Br. abortus in cow's milk, has been demonstrated in this way by many observers, including Fabyan, Evans, Fleischner and Meyer in America, and Zwick and Krage in Germany, and more recently by Wilson and Nutt (1926), Beattie (1932), and Morton (1933) in this country. These are just a few examples amongst many.

The first description of abortus disease in guineapigs is apparently that of Smith & Fabyan (1912)

but a very good account of it, is given by Meyer, Shaw, and Fleischner (1922). These observers noted the following points.

(1) The spleen is always enlarged - and nodular Whilst the other tissues may or may not be affected.

(2) Both Br. abortus and Br. melitensis show a predilection for the sex organs, in both males and females - (Sexotropism) - by whatever mode inoculation is done. (This is very constant and of diagnostic value). They state that ubertropism (predilection for the udders) is only an expression of this sexotropism.

(3) They confirm the disease picture of classical subacute infections, as described by T. Smith and Fabyan, which show - "low mortality rate, pronounced cutaneous hypersensitiveness, positive serum reactions, and distinct lesions in the lymph nodes, spleen, liver, Kidneys, lungs, bones, etc., follow quite regularly the subcutaneous, intraperitoneal, and intratesticular inoculation of infected material or recently isolated cultures.

They quote Robinson (S. Africa 1919) who found that only an enlargement of the spleen occurred with any degree of constancy, and who also concluded that the agglutination test applied to the serums of infected guineapigs is of very great use, especially where the spleen is only slightly if at all enlarged, and not abnormal in appearance.

It should be added that the guineapigs are usually killed 4 to 8 weeks after inoculation, this being the period during which there is most evidence of infection. After about 12 weeks there is a

tendency for the disease to retrogress, and gradual progress is made towards recovery.

The question as to the minimal infective dose of Br. abortus necessary to infect a guineapig has been investigated by Hagan (1922)

Using a 48 hours tube culture, mixed with 2.4c.c. saline as standard, he inoculated a series of guineapigs with successive dilutions of this, from 1 in 10, to 1 in 1,000,000,000. He found guineapigs susceptible even to the smallest dose. Counting the organisms was done, and this dose was reckoned to contain about 9 organisms. He concludes "The infectivity of Br. abortus for guineapigs is extremely great. It is computed that somewhat less than 100 organisms are required to infect most animals".

He also observed that the principal effect on the character of the abortus disease caused by varying the size of the infecting dose, is a change in the time relations. Very small doses produce an infection of slower course than do larger doses, but the ultimate results are practically the same. He also noted that the susceptibility of these animals to infection through ingestion of Br. abortus is relatively slight.

(2) Culture Method.

Various observers have attempted to cultivate Br. abortus from milk, tonsil tissue, and other infected sources, using a variety of methods and media. The guineapig inoculation method is both slow and expensive, and a quicker and less expensive method is very desirable.

Thus Carpenter and Boak (1932) when examining tonsils for Br. abortus, employed as medium infusion agar to which sterile horse serum had been added. They transferred pieces of tonsil tissue or exudate directly to this medium & incubated in 10% CO_2 at 37°C. After 48 hours they picked off and replated any abortus-like colonies.

Poelma and Dickens (1932) in their tonsil investigations used a culture method also, but they do not give any details as to its nature.

The crux of the problem in dealing with such infected material as tonsil tissue, and milk is how to exclude or inhibit the other organisms present, without inhibiting or destroying the Br. abortus. In the case of the tonsils which have such an abundant flora, the problem is particularly difficult, chiefly because of the numerous Gram positive organisms present, Staphylococci, Streptococci, and pneumococci, etc., which rapidly

overgrow any Br. abortus which might be present. Some agent is required which will inhibit the growth of such organisms, without being detrimental to Br, abortus.

The most suggestive work done so far along these lines appears to be that of Huddleson (1920) in connection with milk. This observer, attempted to discover a culture method for isolating Br. abortus from milk.

He employed the dye, Gentian Violet, as the inhibiting agent to check growth of other organisms, incorporating it in the medium (liver infusion agar) to give a final dilution of 1/10,000. By this means he found that a large percentage of organisms occurring in milk other than Br. abortus may be eliminated, and that the dye, when used in above dilution, has no apparent effect upon the growth of Br. abortus. He states that this technique if carefully followed yields results identical with the guineapig inoculation method for determining the presence of Br. abortus in milk. "Its chief advantage is that it requires only 4 days to determine the presence of the organism whereas the animal inoculation method requires at least 8 weeks."

Further observations with this dye were made by Huddleson, Hasley, and Torrey (1927) who note that it inhibits the growth of the majority

of Gram positive organisms, especially fast growing ones, but does not inhibit growth of Br. abortus. Huddleson and Abell (1928) showed that different strains of Br. abortus show a difference in sensitivity to this dye, some withstanding greater concentrations than others. They made the interesting observation that certain dye fast strains (which would not grow on a medium containing 1/100,000 and 1/50,000 of the dye) would, after being suspended in a 1/10,000 solution of the dye for 48 hours, show a luxuriant growth when planted on a solid medium minus the dye.

We quote these observations because of similar results obtained in our own investigations, (using a different dye) showing the great resistance of Br. abortus to certain dyes.

The above observation (Huddleson and Abell) would suggest that it may be an advantage to first cultivate any infected material in broth containing the dye (in proper concentration) and then subculture from this on to a solid medium minus the dye.

In this way strains of abortus more sensitive to the dye may possibly be isolated, which would fail to grow on a solid medium containing the dye. This is the procedure which we employed in our investigations.

II. Br. abortus Infection in Cows.

Infection in cows is diagnosed by finding Br. abortus in the milk or by finding agglutinins in the blood serum.

To estimate the number of infected animals in a herd, the blood serum agglutination test is used, as being most accurate. (Graham & Thorp 1930). To determine the proportion of infected animals which are giving infected milk, the milk must be examined for Br. abortus. We have seen that this proportion varies from 30 to 60%, (page 21). There is apparently no relationship between the agglutinins in the blood and those in the milk. The blood serum may be positive and the milk negative.

The udders are believed to be infected by contact with infected material (genital discharge, switching of tail, etc.) and not from the blood stream. (Cooledge 1916).

Evans found 30% of milk samples in Chicago contained Br. abortus. King & Caldwell found 16% of 151 cows gave infected milk; whilst Carpenter & Boak found 6.1% of 378 cows gave milk containing the organism. Pröscholdt (Germany) records 27.5% positive in 3,000 samples; Beattie (1932) in Edinburgh found 34.9%, and Morton (1933) in Dundee up to 23.6% (raw milk) samples yielded the organism. (by guineapig inoculation). These figures are

much higher than those of Wilson & Nutt (1926) already quoted.

Huddleson, Hasley, and Torrey (1927) showed that the gravity cream contains a far greater proportion of the organisms than any other part of the milk.

III. Examination of Human Blood Sera for Agglutinins to Br. abortus.

(a) from normal people.

Sera sent for Wasserman Test have been used for this purpose, as these people may be regarded as normal in the sense that they had no history or symptoms of Undulant Fever.

McAlpine & Mickle (quoted by Kern 1928), found 0.6% of 10,157 such sera positive to Br. abortus in dilutions 1/100 or over; and Litterer (quoted by Kern) found 1.08% positive. King & Caldwell (1929) found as high as 9% positive out of 1007 sera; Carpenter, Boak, and Chapman record 7.3% of 4,050 sera positive, and 2.4% of 955 sera; Whilst Welsh (1929) gives 5.46% as positive out of 2,433 specimens.

In this country Harrison & Wilson (1928) found 5.5% of such sera positive in dilutions 1/10 or higher; Voge (1929) 6%; and Smith (1932) 4.6% of 1,146 sera in dilutions 1/25 or over.

Huddleson & Johnson (1930) examined sera of 49 Veterinaries and found 57% positive in 1/100 or higher. Only 3 had a history of a disease like Undulant Fever. In these it is an occupational "contact" infection, frequently without overt signs.

(b) from patients with undiagnosed Fever.

These include sera sent for Widal Reaction. Hardy (1928) examined 783 such sera and found 56 positive to Brucella (only 43 were positive to Widal).

Kristensen (1928) in Denmark examined 1177 such sera, and found 89 (=7.5%) reacted to Br. abortus in dilutions 1/100 or over; of these 5 were positive in 1/100; 20 in 1/200; 27 in 1/400; and 18 in 1/800; and 17 in 1/1600 dilution (or higher). Most of these patients showed a clinical condition resembling Undulant Fever, and in 13 out of 20 blood cultures Br. abortus of Bang was isolated (identified by agglutinin absorption and other tests). Two of these 89 cases of "Abortus disease" proved fatal.

Following this discovery all sera in Denmark sent for Widal, were tested for abortus agglutinins, as a routine procedure. As a result 222 cases of Undulant Fever were diagnosed in that country during the first year, and an average of 300 to 400 annually since then. Previous to April

1927 Undulant Fever had not been recognised as occurring in Denmark.

Kristensen argued that such results were not peculiar to Denmark, but would be found in other countries where contagious abortion of cattle is prevalent, if the same procedure were adopted.

We shall see that this has been largely fulfilled.

In this country Harrison & Wilson (1928) found 26% of 42 sera (sent for Widal) positive to Br. abortus (in 1/10 or over); Beattie (1932) found 7 out of 75 positive in "significant titres"; Whilst Smith (1932) had 11 out of 373 sera positive in dilution 1/100 or higher. Messer (1932) records 12 positive out of 186 sera, and 3 out of 4 positive in farm workers.

Interpretation of these Findings.

The general consenus of opinion regards the presence of agglutinins in normal people as being due to active immunity resulting from infection with Br. abortus. The infection is probably in most cases latent, or "subclinical", with no symptoms, or so slight that they are not taken notice of, and result in the majority of cases from ingestion of abortus - infected milk over a prolonged period. The view of Cooledge (1916) that these

agglutinins are due to a passive immunity (simple absorption of agglutinins in milk) is not supported.

The question has been raised as to whether these agglutinins in normal people may be non--specific. Evidence thus far would favour the view that they are definitely specific (Wilson 1930).

The question is also raised - What is to be accepted as a diagnostic titre? Titres from 1/10 to 1/200 have been recommended by different observers.

In Denmark, the minimum titre required for diagnosis of Undulant Fever is 1/100 and this is probably a good average figure. Perhaps the best answer is that given by Wilson (1930) who correlates titre and clinical condition (in 4 groups). This is too long to quote. It has to be borne in mind, however, that cases of Undulant Fever occur with no agglutinins present, and that the height of the titre is not necessarily proportional to the severity of the symptoms.

IV. Abortus Fever (Undulant Fever) in man.

(a) Cases in other countries.

Cases of "Abortus Fever" have been reported from many parts of the world, sometimes sporadic, sometimes in groups.

In the United States, Moore & Carpenter (1926), & Huddleson (1926) reported 12 & 6 cases respectively. In 1927 Evans gives a summary of 20 cases to date.(both bovine & porcine). Hardy (1928) records 83 cases from Iowa State, whilst Sensenich & Giordano (1928) report 7 cases in Indiana. Others report isolated cases from other states. Hardy (1929)(b) makes the following statement, "During 1924 &1925 in the United States exclusive of Texas, Arizona, & New Mexico (where melitensis infection from goats is not infrequent) 10 cases of Undulant Fever were recognised in 6 widely separated States. During 1928, and the first 5 months of 1929 more than 1,000 cases were diagnosed and these had their origin in 42 States". Cattle and swine with contagious abortion were proved to be the source of these infections.

Hardy (1929)(a) analyses a further 125 cases. The clinical features are typical, and most of the patients lived on farms or in country towns. Many cases were occupational (contact infection). Many were porcine in origin. The age incidence fell mostly between 20 and 50 years; none were under 4 years and only 3 were between 5 and 9 years of age.

Similar reports were given by Bierring (1929) (150 cases in Iowa) and by Simpson and Fraizer (1929) (63 cases in Ohio). The latter

found milk and dairy products the most usual source of infection, (i.e. by ingestion).

We have already examined Kristensen's findings in Denmark, and now we see his prophecy with regard to other countries being fulfilled. Dalrymple - Champneys (1929-30) states that in the United States, Denmark, & Sweden, Undulant Fever appears to be a good deal more common than Enteric.

In Germany Poppe (1929) reports 11 cases and quotes 50 others to date. Dalrymple-Champneys (quoting Zeller) gives as many as 626 for Germany between 1st October, 1929, and 30th August, 1930.

Van der Hoeden (1928) reports the first case observed in Holland. In Poland the first 2 cases are recorded by Legezynski (1928) (both veterinaries); whilst Turcu & Morariu (1932) describe the first 2 cases in Roumania (farm workers.)

Goats as a source of infection could be excluded in practically all cases recorded above.

We have omitted reports of cases from Italy & France as melitensis infection of cows is so frequent in those countries. But in some districts of France as many as 80% of cases are traced to sheep (melitensis).

We have seen that infection could

apparently take place through the skin - "contact infection", and occurred in veterinaries, slaughterers, packing house workers and others who handle infected material. (Hardy 1929). This was substantiated by the experiments of Hardy, Hudson, & Jordan (1929) who, using guineapigs, showed that infection occurs much more readily through the skin (whether intact or abraded), than via the mouth (ingestion).

The skin acts also as a portal of entry for Br. melitensis, as seen in laboratory workers, who frequently get infected from handling cultures.

(b) Cases in this country.

Byam (1918) & Box & Bamforth (1925) record 1 and 2 cases respectively of Undulant Fever originating in England; but both were traced to goats' milk, and therefore possibly due to Br. melitensis.

Bamforth (1927) was the first to record 2 cases in England in which a bovine source of infection (cow's milk) was suspected, though not proved. One was a woman 63 years of age, and the other a man aged 40. The serum of both agglutinated Br. abortus up to a dilution of 1/2000, and Br. melitensis up to 1/1,000. Both had symptoms of Undulant Fever.

Wordley (1927) records 2 further cases of

typical Undulant Fever of uncertain source. Thomson (1928) describes a case (first diagnosed as Malaria - -but tests for this negative) - in which Br. abortus was agglutinated to 1/1,024, and Br. melitensis to 1/512. He was a farmer; some of his own cows had contagious abortion and he had been using the milk.

Thereafter a number of isolated cases is recorded by various observers, including Manson-Bahr (1928); Davies & Anderson, Charles & Warren, Todd, Bloxsome & Davey, & Nightingale & Wilson - all in 1929. The last named were the first to isolate the organism by blood culture. It was thoroughly tested and identified as Br. abortus. In Todd's case it was isolated from the urine.

In 1930 McArthur & Wigmore (urine culture positive), Williams & Sladen, J.N. & R. Cruickshank, (first case in Scotland), Wishart & Gibson, & Scott, all report one case each.

Similarly in 1931 & 1932; Smith (1932) reports 10 cases from the North East of Scotland.

According to Mackie (1933) 136 recorded cases of Abortus or Undulant Fever have been collected in the British Isles since 1925.(25% of them being in Scotland). Wilson reckons from the ratio of Abortus Fever to Typhoid that there must have been about 480 cases in Britain in 1930. If this represents an average annual incidence, there

must be many cases unrecognised and unrecorded.

At the same time, as clinical consciousness of the disease becomes more wide spread amongst medical men, and as routine laboratory methods are more generally adopted, an increasing number of cases is brought to light in various countries. It would appear that such cases must formerly have been diagnosed as Influenza, Tuberculosis, Typhoid, Paratyphoid, sometimes as Appendicitis; or Glandular Fever, or simply as Pyrexia of Unknown Origin.

V. Br. abortus Infection of Reproductive Organs (including human abortion)

As both Br. abortus and Br. melitensis show a predilection for these organs (sexotropism) in animals, with abortion as the chief symptom, investigators have been on the look out for similar manifestations of infection in the human subject.

This has been referred to already (page 24)

Among earlier reports de Forest (1917) mentions 11 cases of abortion in women, where the source of infection was believed to be cattle infected with Br. abortus. Carpenter & Boak (1931) examined 28 foetuses and 34 placentas and isolated Br. abortus (bovine) from 1 foetus. Kristensen & Holm (1929) isolated the organism from the placenta in 1 case of abortion during Undulant Fever. They

consider that there is a great tendency to abortion in women who have this Fever.

Simpson & Fraizer (1929) observed 5 women (with negative Wasserman) who had aborted repeatedly and gave histories of undiagnosed fevers. Their titres ranged from 1/80 to 1/320 with an abortus antigen. All were drinkers of raw milk.

Dalrymple-Champneys (1932) regarded it as "now proved" that Br. abortus infection can cause abortion in women, and that the organism has been recovered from the uterine discharge, placenta, and foetus in several cases.

Apart from pregnancy Br. abortus has been isolated in a few gynecological conditions, but this would appear to be somewhat rare.

Infection of the male organs with Br. abortus has been reported (Simpson & Fraizer 1929). Orchitis occurs as a complication of Melitensis Undulant Fever.

But speaking generally in both man and animals the male organs are apparently much less susceptible to Br. abortus infection than are the female organs (especially if pregnancy supervenes)

VI. Br. abortus Infection in Children.

We have already noted that Br. abortus

infection, clinically recognisable, is not common in children.

Thus Hardy (1928) amongst 83 cases found none under 7 years of age, and (1929) amongst 125 cases none were under 4 years, and only 3 were between 5 and 9 years. He suggests that children may have a high degree of immunity, or have acquired the disease in a mild form. Evans (quoted by Voge 1929) suggests that cases of obscure Fever which occur frequently in infants may be due to an infection with Br. abortus acquired from the consumption of unpasteurised cow's milk. Such children would show agglutinins in their sera for some time afterwards. It is interesting to note that the highest proportion of positive sera recorded (normal people) is actually in children, namely 17% (Larson & Sedgwick 1913).

Simpson & Fraizer (1929) observe that the relative immunity in children appears to parallel the immunity seen in calves. Carpenter (1924) conducted investigations regarding Br. abortus invasion of the tissues of calves from the ingestion of infected milk. He found that the lymph nodes draining the mouth and pharynx became infected first and remained infected the longest of any of the tissues examined; and he concluded that in experimental and domesticated animals the infection localised in lymph nodes and lymphoid tissue, frequently producing a focal or general lymphadenitis.

He frequently recovered Br. abortus in pure culture from the lymph glands, and he concludes that this organism has an affinity for lymph or lymphoid tissue. His observations suggested a study of tonsils in man, since these are lymphoid structures, and may be the actual portal of entry of Br. abortus into the human organism, when infected milk is ingested. From the tonsils the lymph nodes may become infected (latent infection). In connection with this, Wilson (1929-30 Discussion) suggests that in order to ascertain the extent of this latent infection it might be advisable to examine the lymphatic glands of a series of children dying from various causes.

Manson - Bahr & Willoughby (1929) also draw attention to the spread of Br. abortus and Br. melitensis by the lymphatic route, producing a specific adenitis. They consider that this is not sufficiently recognised. They found cervical adenitis in 3 out of 6 cases.

One would imagine that the tonsils from their exposed position and structure (numerous crypts) might be readily infected with Br. abortus, when infected milk is ingested.

This suspicion is further strengthened by the fact that at least 3 different observers have been successful in isolating Br. abortus from tonsil tissue.

Mohler & Traum (already quoted) found 1 positive in 56 Tonsils and Adenoids examined. Poelma and Dickens (1932) investigated 116 pairs of tonsils and isolated Br. abortus from 2 pairs. Carpenter & Boak (1932) cultivated the organism from 8 of 56 pairs. It was found that these 8 positive cases had been drinking milk infected with Br. abortus, and 1 case was suffering from Undulant Fever. Two of the 8 were children 4 years of age. The authors conclude that these 8 Tonsils were a probable focus for the infection.

King (quoted by these last observers) examined 49 pairs of Tonsils, but found them all negative for Br. abortus.

We conclude from the above reports that the tonsils do in some cases become infected with Br. abortus.

We decided to carry out a similar investigation in tonsils from children and our reasons for so doing will be discussed more fully in Part II.

Part II. THE PRESENT INVESTIGATION.

Introduction.

So far as we are aware all investigations on the subject of Br. abortus in Tonsils appear to have been carried out in America. These have already been referred to under review of the literature. We have not found any record of similar research having been done in this country.

It appeared that it might fill a gap in our Knowledge to examine a series of tonsils taken from children only, on the hypothesis that these organs may be either a portal of entry or an actual seat of latent (focal) infection for Br. abortus. "The organism may invade the tonsil and multiply or accumulate there until the resistance of the host is decreased from fatigue or from disease, permitting its invasion of the blood stream." (Carpenter). From reports already quoted it would appear that the tonsil is sometimes such a seat of focal infection with this organism.

We confined our examination to tonsils from children, especially those of 5 years and under, as they presumably consume a larger proportion of milk than any other age group. Tonsillectomy is also a much more frequent operation in children, and so there is no lack of material to work with.

We selected the cases who gave a history of using raw cow's milk, either wholly or partly, and excluded as far as possible those using only pasteurised milk. In some cases both kinds were used, but in a certain proportion there was uncertainty as to the nature of the milk used. Most of these probably used some raw milk, judging by enquiries made at the time (See Table I).

We preferred to include some children between 6 to 10 years of age, who were using raw milk, rather than include younger ones who were having only pasteurised milk.

The investigation was carried out between November 1932 and May 1933 and altogether 100 pairs of Tonsils were examined; 72% were from children 2 to 6 years of age (none were under 2 years) and 28% were from children between 6 and 10 years.

The tonsils were obtained from the Surgical Out Patient Department, Royal Infirmary, Dundee, through the courtesy of Mr. R.P.Mathers, and Mr.M.J. Gibson of the Ear, Nose, and Throat Department.

The patients were having tonsillectomy done because of the usual symptoms of enlarged or diseased tonsils (and adenoids). They were in all cases removed by guillotine. Adenoid tissue was not included in our investigations.

The tonsils were received directly into a

sterile Petri dish, and conveyed to the laboratory.

TABLE I.

	Using raw milk	Using Pasteurised milk	Using Milk of Uncertain Source	Total
2-6years	37	6	29	72
6-10years	18	none	10	28
	55	6	39	100

These figures represent percentages since 100 were examined.

Table I is inserted to make more clear the childrens' age groups and the kind of milk they were using. Regarding the 3rd. column ("uncertain source") we were satisfied, from enquiries made, that these were using some raw milk.

Methods Used.

Two methods were used in our attempt to isolate Br. abortus from tonsil tissue.

(1) Guineapig Inoculation.

(2) A Culture Method.

1. Guineapig Inoculation Method.

Preliminary Experiments.

Before this could be proceeded with satisfactorily 2 preliminary problems had to besolved, namely, to find;-

(1) the best method of preparing the tonsil tissue for inoculation.

(2) the optimum dosage of this tissue.

The first batch of 6 tonsils were prepared as follows:- Each pair of tonsils was transferred from the petri dish, cut up in a mortar with scissors, and ground up with 5cc. saline (everything being sterilised). This was then centrifuged and 20 Minims of the supernatant fluid was injected (subcutaneously in the abdomen) into each of 6 guineapigs. Three of these animals died within 4 days, and it was found that pneumococcal infection was the predominating factor in each case.

We then decided to try the effect of protecting the guineapigs with Antipneumococcal serum. Ic.c. of this was given 24 hours before inoculating the tonsil tissue. Control animals received tonsil tissue but no serum.

Also a smaller dose, 10 Minims, of supernatant fluid was tried. Some animals were also inoculated with the tonsil tissue residue underlying the supernatant fluid. This, however, was difficult to inject, containing rather too much solid tissue; whilst the supernatant fluid appeared to be too much the reverse.

Technique Adopted.

We then tried mincing up the tonsils (in a mortar with scissors as before), grinding up with 5cc sterile broth, and then filtering all this

through a double layer of sterile gauze,(no centrifuging). This preparation proved very satisfactory, the filtrate being used. This is referred to hereafter as the gauze filtrate and was used through out the remainder of our experiments. Pairs of tonsils were used everytime.

The optimum dose of this filtrate was found to be 0.25cc. injected subcutaneously into the abdominal wall. Larger doses were apt to cause septic infection; sometimes fatal. This was therefore adopted as our routine procedure throughout; one guineapig being inoculated for every pair of tonsils.

As control animals receiving no protective anti-pneumococcal serum did well with this technique, the serum was early discontinued.

Examination of Guineapigs

The animals were killed after 6 to 8 weeks and autopsied. (As many as possible were kept the full 8 weeks as we wished to note the incidence of Bacillus tuberculosis in the tonsils, whilst examining for Br. abortus).

Any macroscopic lesions were noted-and specially looked for in spleen, lymph nodes, and sex organs.

The spleen was removed, the surfaced seared

and the pulp inoculated on to suitable medium. This was done in every case, whether the spleen looked normal or otherwise.

The guineapig's blood was collected and centrifuged to separate the serum, and this was examined for agglutinins to Br. abortus. This was also carried out in every case irrespective of naked-eye appearance.

The medium we used for cultures from spleen consisted of Agar plus Oxheart extract plus Fresh rabbit blood (10%) plus glucose 1%; heated at 85°C for 15 minutes. It was of a chocolate colour, and resembles "Chocolate Agar" in composition.

With this special medium it was hoped to facilitate isolation of Br. abortus if only present in small numbers. This medium is referred to hereafter as "Special Medium". When growing on this Br. abortus showed characteristic tiny "seed pearl" colonies.

Tube slopes were used and these were incubated at 37°C in a glass jar from which 10% of air was exhausted, and replaced by CO_2 from a Kipp's apparatus. Cultures were kept as long as 10 to 14 days in case of delayed growth occurring.

The serum agglutination test was carried out in dilutions of 1/50, 1/100, 1/200, 1/400, and 1/800 as a routine. The antigen used was a stock

culture of Br. abortus.

The tubes containing serum and antigen were incubated at 55° C for 4 hours, after which the first reading was done; the second being made the following morning, i.e. after standing at room temperature over night.

RESULTS.

The result was entirely negative throughout the whole series. In no instance did we find a guineapig inoculated with tonsil tissue, develope any signs of Abortus infection. Both cultures from the spleen, and serum agglutination tests, were negative in every case.

11. Culture Method.

We decided to make use of Crystal Violet to inhibit Gram positive organisms of the Tonsils. This dye was used by Haxthausen (1927) to inhibit Staphylococci when investigating certain skin diseases. Kinnear (1931) also used the same method, and a weak concentration 1/500,000 was found effective under these conditions for inhibiting growth of Staphylococci, but allowing growth of Streptococci (in skin conditions).

It was necessary here also to carry out

preliminary experiments in order to find out:-

(1) what concentrations of the dye gave best results.

(2) what amount of tonsil tissue proved most suitable.

(3) what length of incubation would prove most satisfactory.

Preliminary Experiments

In our first experiment we employed a series of test tubes containing each 10cc broth to which Crystal Violet was added to make the following concentrations: 1/100,000, 1/200,000, 1/400,000, and 1/800,000. To each of those a loopful of tonsil tissue was added (tonsil residue left after centrifuging), from 3 different tonsils, making 3 series of 4 tubes, 1 series to each tonsil. Control tubes, without Crystal Violet, were also included (broth only).

These Crystal Violet broths plus tonsil tissue were incubated for 48 hours at 37°C in a jar containing 10% CO_2 atmosphere. Films were then made and stained with Gram's stain. It was found that all contained Gram positive cocci, including streptococci, staphylococci, and pneumococci.

The flora was very mixed (Gram negative plus Gram positive) but Gram positive tended to diminish as dye increased.

These concentrations of Crystal Violet

were therefore not sufficiently strong to inhibit the Gram positive organisms.

The above experiment was accordingly repeated using Crystal Violet in the following dilutions - 1/10,000, 1/20,000, 1/40,000, and 1/80,000. The same 3 tonsil preparations were used, (having been kept in ice-chest). Films were examined after 3 days incubation. It was found that the strongest concentrations of Crystal Violet 1/10,000 and 1/20,000 inhibited the Gram positive organisms, whilst the 2 weaker strengths failed to do so.

This test was repeated using another 3 tonsils; and after 5 days exposure to Crystal Violet subcultures were made on ordinary agar and Gram positive organisms found present in all.

Crystal Violet in Concentrations of 1/5000, 1/7,500, 1/10,000, 1/20,000, and 1/40,000 were then used, and it became apparent that the results were influenced very much by the amount of tonsil tissue present. The more tissue present the more would the effect of the dye be reduced or weakened.

It was found that the best results were obtained with Crystal Violet in concentrations of 1/7,500, and 1/10,000, when to these were added a measured quantity of the gauze filtrate tonsil preparation, namely 0.2cc. With these adjustments

Gram positive organisms were for the most part inhibited (though not entirely) whilst Gram negative organisms, (bacilli) survived and could be subcultured.

The following Tables and notes are submitted to make these findings more clear.

A batch of tonsils, numbers 19 to 24, were prepared (gauze filtrate) and inoculated into 6 series of Crystal Violet broths of following concentrations. (0.2cc gauze filtrate to each).

a = 1/5,000 Crystal Violet.
b = 1/7,500 " "
c = 1/10,000 " "
d = 1/20,000 " "
e = 1/40,000 " "

After 5 days incubation in 10% CO_2 at 37°C. the degree of Decolourisation in each tube was noted, and is shown in the following table.

TABLE II.

Crystal Violet / Tonsil Number.	a 1/5000	b 1/7500	c 1/10000	d 1/20000	e 1/40000
19	++	++	++	++	++
20	+−	++	+	++	++
21	+−	++	+	++	++
22	−	−	+−	++	++
23	+−	++	++	++	++
24	−	−	+−	++	++

N.B. ++ = complete decolourisation
+ = marked "
+− = slight "
− = no "

From this Table and results in Table III.

we concluded that the concentrations a, d, and e, might be dispensed with in future, and that "b" and "c" would be quite sufficient and satisfactory for our purpose. "b" and "c" seemed to represent the borderline between too much and too little decolourisation. Slight differences in amount of tonsil tissue present would no doubt help to account for some variations, also the different numbers and kinds of organisms present in different tonsils.

We selected a dozen of these and subcultured to ordinary agar and Fildes agar, and examined the resulting growths for presence of tiny Gram negative bacilli (which we were searching for) and for Gram positive organisms (which we were trying to exclude). The results of these subcultures are given in Table III. (growth after 48 hours).

The results in Table III. include naked-eye and microscopic examination of the cultures. The term "Influenzoids" includes all tiny Gram negative bacilli which might be either Influenza group bacilli; or Br. abortus, or organisms closely resembling these. The colonies were mostly transparent or translucent. Those growing on Ordinary Agar were of special interest as the possibility of their being Br. abortus had to be considered - 4 tonsils yielded these namely Numbers 20, 21, 23, and 24. On further subculture to Ordinary agar 23, and 24 did not grow, and it was concluded that those were probably "True Influenzoids"

TABLE III.
Tonsils 19 - 24
after 6 days in Crystal Violet.

Subcultures on (1) Ordinary Agar and (2) Fildes Agar Slopes.
Findings after 48 hours subculture.

Ordinary Agar (12tubes)		Fildes Agar (12tubes)
	19a	
Gram positive cocci only	(decolourised)	Gram positive cocci only
	20a	
Gram positive cocci only	(Partial) (decolourisation)	Gram positive cocci & Influenzoids
	20b	
Influenzoids	(decolourised)	Influenzoids
	21a	
Gram positive cocci & Influenzoids	(Partial) (decolourisation)	Gram positive cocci & Influenzoids
	21b	
Gram positive cocci Influenzoids	(Completely) (decolourised)	Gram positive cocci Influenzoids
	22b	
Gram positive cocci Larger pleomorphic Gram negative bacilli	(No) (decolourisation)	Pleomorphic Gram negative bacilli (too large for Influenzoids)
	22c	
Gram positive cocci	(Partial) (decolourisation)	Gram positive cocci Influenzoids
	23a	
Gram positive cocci Influenzoids	(Slight) (decolourisation)	Gram positive cocci Influenzoids
	23b	
Gram positive cocci & Influenzoids	(Complete) (decolourisation)	Gram positive cocci & Influenzoids
	24b	
Gram positive cocci Influenzoids	(No) (decolourisation)	A few Gram positive cocci
	24c	
Gram positive cocci only	(Partial) (decolourisation)	Gram positive cocci & Influenzoids
	24d	
Gram positive cocci only	(Complete) (decolourisation)	Gram positive cocci & Influenzoids

Ordinary Agar 4 Influenzoids out of 6 Tonsils.

Note The Decolourisation in Table III. is taken from Table II. and included here for convenience.

Fildes Agar 5 Influenzoids out of 6 Tonsils.

which had survived the first subculture to Ordinary Agar because presumably a small amount of tonsil tissue (with "X" and "V" factors) had been transferred from the Crystal Violet broths. 20, and 21 grew well, however, on Ordinary agar on repeated subculture, both in 10% CO_2 and in air; and we were able to isolate them in pure culture and submit them to the agglutination test against abortus antiserum. These were the first examples of Gram negative organisms isolated from tonsils by use of Crystal Violet which might possibly be Br. abortus. As we shall see we succeeded in several other instances in isolating similar organisms.

The isolation of these True Influenzoids was also of great interest, and it was suggested that we might examine all our tonsils for these organisms, with a view to finding out how often B. influenzae (Pfeiffer) can be found. This would form a useful subsidary part of our investigation. This is dealt with in Section II. of this thesis.

As a further example of the use of this Crystal Violet technique for isolation of tiny Gram negative bacilli which might be abortus we submit Table IV. The technique was the same only the C.V. broth plus Tonsil cultures were incubated 6 days (instead of 5) before subculture. The next batch of tonsils Numbers 25 - 30 were used.

TABLE IV.

Subcultures 6th day from Crystal Violet on to Ordinary Agar, examined after 48 hours incubation in 10% CO_2.

Crystal Violet "b"	Crystal Violet "c"
Tonsil 25.	
Heavy growth, mostly small colourless colonies. Mic:(1) Colourless=tiny Gram negative bacilli (2) opaque=Flavus.	Heavy growth, opaque and small colourless colonies (1) thin background of pneumococci and tiny Gram negative bacilli. (2) opaque Flavus.
Tonsil 26.	
Small colourless and tiny opaque - poor growth. (1) Colourless=Gram negative bacilli (2) Tiny opaque=Gram positive cocci	Heavy growth-large opaque and smallshiney colonies. (1) small shiney = Gram negative cocci (2) large opaque = Flavus.
Tonsil 27.	
poor growth Streptococcal in type Mic: Pneumococci only.	poor growth Streptococcal in type Mic: Pneumococci only
Tonsil 28.	
Fair growth - large transparent, and small Streptococcal type Mic: Pneumococci and Leptothrix	Fair growth - as in "b" Pneumococci or Streptococci.
Tonsil 29.	
Slight growth Colonies irregular in outline Mic: Leptothrix (pure culture)	Slight growth Streptococcal type Pneumococci or Streptococci.
Tonsil 30.	
No growth	Heavy growth - large opaque-and Small colourless. Mic: shows only Gram negative cocci apparently Flavus.

In Table IV. we note that Gram negative bacilli were found in only 2 out of the 6, namely Numbers 25 & 26. (25b, & c. & 26b.). On further subculture to Ordinary agar 25 died out, therefore probably a true haemoglobinophil. But Number 26 grew on repeated subculture, and with the aid of Penicillin agar 1/10 a pure culture was obtained. In the same way, of the next batch of tonsils (Numbers 31 - 36), one, Number 32, yielded a similar tiny Gram negative bacillus, which grew well on Ordinary agar. These 2 organisms, along with Numbers, 20, and 21 (already mentioned), were the first 4 strains thus isolated, which might possibly be Br. abortus. These formed the first batch submitted to the agglutination test.

Technique Adopted.

These concentrations and dosage were established therefore as a routine procedure throughout the rest of our investigations, viz:

(1) using only 2 strengths of Crystal Violet 1/7,500 and 1/10,000, i.e. "b" & "c".

(2) as dose of inoculum - 0.2cc gauze filtrate for each tonsil examined.

(3) Regarding the length of incubation in Crystal Violet,- 5 days seemed to be about the optimum, although any period from 4 to 7 days gave usually little difference in results. We adopted 5 days as a routine.

It still remained to find out whether Br. abortus would withstand these somewhat high concentrations of Crystal Violet. This was done by subsidiary experiments to be described later.

Our routine procedure thus evolved therefore was:-

(1) to take 2 tubes each containing 5cc broth - (for each pair of tonsils). Add Crystal Violet to each to give dilutions 1/7,500, and 1/10,000 respectively.

(2) Add to each of these 0.2cc gauze filtrate from each pair of tonsils (mixing thoroughly).

(3) Incubate these at 37°C in a jar containing 10% CO_2 atmosphere for 5 days.

(4) then subculture from Crystal Violet tubes to ordinary agar plates.

(5) incubate these plates for 2 to 3 days (usually 3) in the same jar.

(6) examine the plates for the presence of any organism resembling Br. abortus.

Regarding the last stage the plates were first examined Naked-eye. A film was made from any suspicious or doubtful colonies, stained with Gram, and examined microscopically. If a Gram negative organism was found bearing the least resemblance to Br. abortus - a subculture was made (usually to Ordinary agar tube) to preserve the culture, and the organism was tested against abortus antiserum.

Various organisms were found growing on these plates, - including Gram positive organisms sometimes, and it was occasionally difficult to get a pure culture of any Gram negative bacillus which we wished to isolate.

In such cases we subcultured to Penicillin agar 1/10 for 24 hours or longer, to get rid of the

Gram positive organisms, and then back to Ordinary agar.

This procedure was carried out for the whole series of 100 pairs of tonsils. In the majority we failed to isolate any organism resembling Br. abortus. In 14% however, tiny Gram negative bacilli were isolated by the technique described, though sometimes it was very difficult to procure a pure culture. We found that Gram positive organisms were more resistant to Penicillin agar than to Penicillin broth - (in same concentration 1/10) - when used to procure pure cultures.

These 14 abortus-like strains were all tested against abortus antiserum, using normal rabbit serum as a control. 8 were negative to the abortus antiserum; the other 6 gave a positive agglutination reaction to both abortus antiserum and normal rabbit serum. Dilutions from 1/50 to 1/3,200 were employed.

Results and Conclusions.

(1) The dye, Crystal Violet has an inhibitory effect upon Gram positive organisms, whilst allowing growth of small Gram negative bacilli - at least in the presence of Tonsil tissue. The optimum concentrations of Crystal Violet for isolation of these bacilli from tonsils were

1/7,500, and 1/10,000.

(2) Small Gram negative bacilli were by use of the Crystal Violet Technique described, isolated from 14 out of 100 pairs of tonsils. These were capable of growing on Ordinary agar - thus not behaving like B.. influenzae (Pfeiffer).

(3) These 14 strains were all submitted to the agglutination test against abortus antiserum - controls with normal rabbit serum being used in every case. Serum dilutions from 1/50 to 1/3,200 were used; but in every case the test proved negative for Br. abortus. Six of the 14 gave a positive reaction with the abortus antiserum, but also gave a similar reaction with normal rabbit serum.

(4) The results therefore of our examination of 100 pairs of tonsils for Br. abortus by Culture Method were completely negative; the same as obtained by the guineapig inoculation method.

Subsidiary Experiments in Br. abortus investigation.

The object of these experiments was twofold.

(1) to test the resistance of Br. abortus to the dye Crystal Violet, both in presence and absence of Tonsil tissue.

(2) to test the resistance of Br. abortus to Penicillin (which was recommended by Fleming for inhibiting Gram positive organisms).

I. Resistance of Br. abortus to Crystal Violet.

This was tested in the following ways:-

(A) Qualitatively in presence of tonsil tissue.
(B) Quantitatively in " " " "
(C) Qualitatively in absence " " "

Experiment A. (Qualitative).

Two tonsils were used, Nos. 10 & 11 and 3 concentrations of Crystal Violet in broth (10cc)

a = 1/10,000
b = 1/20,000
c = 1/40,000

and a control (broth only) for each.

1 loopful of tonsil residue (No. 10 & No. 11) and 1 loopful Br. abortus emulsion were added to each tube i.e. 4 tubes for No. 10 tonsil, and 4 for No. 11 (N.B. The Br. abortus emulsion was prepared by washing off 24 hours growth (special medium) tube 5" x 5/8" in 1 cc broth.).

These were incubated 4 days in 10% CO_2, then subcultured to tubes of same medium, and incubated 3 days in 10% CO_2. These slopes were then examined naked-eye and microscopically. In 10a & 11a seed-pearl

colonies were present, which on microscopic examination showed tiny Gram negative bacilli. Tested against abortus antiserum (in dilutions 1/50 to 1/3,200) these were proved to be Br. abortus. From the other (weaker) concentrations of Crystal Violet Br. abortus was not isolated, though it may have been present in small numbers. Gram positive organisms predominated in these.

Conclusions from Experiment A.

(1) Br. abortus is able to survive (and grows on subculture) after 4 days incubation in 1/10,000 Crystal Violet i.e. in the presence of (a small amount of) tonsil tissue.

(2) This same concentration of Crystal Violet has a definite inhibiting effect upon the Gram positive organisms present.

(3) Weaker concentrations of Crystal Violet allow Gram positive organisms to survive and grow in more or less profusion.

(4) This result for Br. abortus is qualitative only.

Experiment B. (Quantitative).

This Experiment is divisible into 2 parts which we will call B1 and B2. The object of these was to find out how Br. abortus survives (or grows)

(1) When various doses of Br. abortus are used as inoculum.

(2) When various concentrations of Crystal Violet are used (in broth).

Experiment B1.

Tonsil gauze filtrates of Three tonsils

Nos. 16, 17, & 18, were used, and 5 different concentrations of Crystal Violet namely:-

a = 1/5,000
b = 1/7,500
c = 1/10,000
d = 1/20,000
e = 1/40,000

were used for each, in tubes containing each 10cc broth.

It is to be noted that stronger concentrations of Crystal Violet are included here than were used in Experiment A.

The Br. abortus was prepared as follows:- A 24 hours culture (Special medium slope 5" x 5/8") was washed off in 1cc broth. This was taken as standard or unity.

Of this Br. abortus emulsion.

A dilution of 1/10 was added to Tonsil filtrate No.16
" " " 1/100 " " " " " No.17
" " " 1/1,000 " " " " " No.18

Of each of these mixtures (Tonsil filtrate + Br. abortus) one loopful was added to each of 5 Crystal Violet broth tubes - i.e. one series of above 5 different concentrations of Crystal Violet for each tonsil.

These were incubated in 10% CO_2 at 37°C for 5 days, then subcultured to Ordinary agar and Fildes agar plates, which were incubated for 3 days (under same conditions). On examination we found a tiny Gram negative bacillus growing in 16e & 17e

plates (on both Ordinary agar and Fildes agar) i.e. from Crystal Violet concentrations 1/40,000 (weakest used). By agglutination reaction this organism was identified in both cases as Br. abortus.

Some Gram positive organisms were also present on 16e & 17e plates, whilst others showed both Gram positive and Gram negative ones; A good number from the stronger Crystal Violet concentrations (a,b, and c) showed no growth at all. We could not retrieve Br. abortus from No. 18, series nor from any others except 16e & 17e.

We have to bear in mind that the amount of tonsil tissue present in this Experiment is very small (1 loopful).

Experiment B2.

Three tonsils were also used in this experiment Nos. 25,26,& 27 - gauze filtrate of each being prepared as usual. Br. abortus was added to each in progressively smaller doses than in the last experiment, so that this is an extension of Experiment B1 so far as Crystal Violet is concerned. But only 2 concentrations of Crystal Violet were used,

1/7,500 = b
1/10,000= c

(the same as we had adopted in our routine investigation (p 65). In addition we also employed 2 concentrations of Penicillin (in broth)(1 in 2½, & 1 in 5) inoculating them in the same way with the mixture of Tonsil

filtrate and Br. abortus. This was done so that we might be able to compare the resistance of Br. abortus to Crystal Violet and Penicillin.

The Br. abortus emulsion was prepared as in Experiment B1, except that 5cc broth were used this time (instead of 1cc) in washing off the culture (the other details being the same). The emulsion here was therefore only 1/5 the strength of that of B1. This was compensated for by the fact that in the present Experiment we employed a larger dose of inoculum, 0.1cc of the mixture (Tonsil filtrate + Br. abortus) being added to every tube.

Br. abortus was added to each of the 3 Tonsil filtrates in the following concentrations:-

A dilution of 1/10,000 was added to No. 25 Tonsil filtrate
" " " 1/100,000 " " " No. 26 " "
" " " 1/1,000,000 " " " No. 27 " "

Taken along with Experiment B1. we have therefore a series of dilutions of Br. abortus ranging from 1 in 10 to 1 in 1,000,000, all tested against Crystal Violet. In the second series, however, (25, 26, & 27) there is an increase in the amount of tonsil tissue present.(0.1cc gauze filtrate).

In Experiment B2. we have thus 4 tubes inoculated with each Tonsil filtrate + Br. abortus, i.e. 2 containing Crystal Violet ("b" & "c") and 2 containing Penicillin (1/2½, & 1/5) = 12 tubes in all. These were incubated in 10% CO_2 at 37°C for 4 days,

then subcultured to Ordinary agar plates and incubated for 2 days under same conditions. The plates were then examined for presence of Br. abortus.

The results of this Experiment are given in Table V.

It will be seen that tiny Gram negative bacilli were grown from all 3 of the Mixtures (Tonsil filtrate + Br. abortus) added to the Crystal Violet broths. In 26 they grew from the weaker concentration (1/10,000) only. As they were not in pure culture (Gram positive cocci etc., being present), subcultures were made from the Ordinary agar plates, to Penicillin agar (1 in 10) (Fresh Penicillin) using Crystal Violet "b" in case of 25, Crystal Violet "c" in 26, & Crystal Violet "b" in 27. After 24 hours incubation minimal growth was present in all 3 and in all instances the organism (on Microscopic examination) proved to be filamentous in shape, and some even showed great swellings upon them. However, a "rough" agglutination test showed that these aggluntinated with Abortus antiserum.

The tubes were subcultured back to Ordinary agar as the Gram positive flora had disappeared through subculture on Penicillin agar. But the bizarre morphology of the Br. abortus on Penicillin agar (1 in 10) suggests that it does not like Penicillin and might die out unless subcultured.

TABLE V.
EXPERIMENT B2

Tonsils 25, 26, & 27, + Br. abortus.

after 4 days in Penicillin and Crystal Violet; Subcultures to Ordinary Agar Slopes; after 48 hours.

Penicillin "A"	Penicillin "B"	Findings	Crystal Violet "b"	Crystal Violet "c"
		25 + Abortus		
Heavy growth opaque colonies Colourless colonies like B. influenzae	same as "A"	Naked-Eye	Light growth Streptococcus Colonies A few ?Influenzoid colonies	same as "b"
		Microscopic		
Gram — cocci ###	Gram — cocci ### Gram — bac. few Gram + bac few (diphtheroids)		Gram — bac ### tiny Gram + cocci tiny	Gram — bac ### abortus-like. Leptothrix ##
A	B	26 + Abortus	"b"	"c"
Heavy growth opaque colonies Flavus colonies Colourless colonies	same as "A"	Naked-Eye	no growth	slight growth Large colourless colonies.
		Microscopic		
Gram — cocci ### Gram — bac. few (large)	Gram — cocci ### Gram — bac. +		nil	Gram — bac ### tiny
A	B	27 + Abortus	"b"	"c"
Heavy growth Flavus colonies Few colourless colonies	same as "A"	Naked-Eye	Fair growth Colourless Colonies	Fair growth in condensation water
		Microscopic		
Gram — cocci ### Gram — bac +	Gram — cocci ### Gram — bac few (tiny ones present)		Gram + cocci ## Gram — bac ### tiny	Gram + cocci + Gram — bac ### tiny

N.B. Penicillin "A" = 1 in 2½ (broth) Crystal Violet "b" = 1/7,500
" "B" = 1 in 5 (") " " "c" = 1/10,000

Gram + = Gram positive Gram — = Gram negative bac. = bacilli

The signs + ## ### denote different degrees of growth.

The subcultures to Ordinary agar showed a normal abortus-like organism (Microscopically), in all 3 cases, and on examination against abortus antiserum in dilutions 1/50 to 1/3,200 they were all identified as Br. abortus.

Regarding results with Penicillin (See Table IV) on subculture, the predominant organism grown was in every case a Gram negative coccus of the Flavus type, whilst Colourless Influenza-like colonies were also present. A few Gram negative bacilli were seen (Microscopically)(tiny, but few, in one case(27)) but we could not isolate Br. abortus from any of these. The impression we gained referred to above, is thus confirmed, namely, that Br. abortus appears to be sensitive to Penicillin. This investigation is pursued further in Experiment D.

The Penicillin here used was weaker than standard (14 days culture instead of 7) - hence strong concentrations used (1/2½ and 1/5). In Experiment B.2.

Conclusions (from Experiments B1 & B2)

1. When inoculated into Crystal Violet broth (concentrations 1/7,500, & 1/10,000,) in minimal dosage (one millionth of a 24 hour tube culture 5" x 5/8" on special medium) and incubated for 4 days, Br. abortus survives and can be isolated from such cultures - that is, in the presence of a small amount of tonsil tissue. This suggests that Br. abortus shows great resistance to this dye.

2. The question arises - would Br. abortus behave similarly towards Crystal Violet in the absence of tonsil tissue? This suggests further investigation along this line - adding pure cultures of Br. abortus, to various Crystal Violet broth concentrations. This is carried out in Experiment C.

3. We may also conclude that our technique for attempted isolation of Br. abortus from tonsil tissue should be satisfactory, even if Br. abortus is present only in minimal amount. i.e. using Crystal Violet broth in 1/7,500, & 1/10,000 concentrations, and adding 0.2cc Tonsil filtrate (more tonsil tissue than in these experiments).

4. When exposed to Penicillin agar 1 in 10 Br. abortus after 24 hours shows a bizarre morphology. (filamentous with curious swellings). When inoculated into Penicillin broth 1 in 2½ and 1 in 5 in the doses used in our Experiment - and incubated 4 days, Br. abortus cannot be retreived (i,e, in presence of small amount of tonsil tissue). This suggests that Br. abortus is sensitive to Penicillin, but further experiment along these lines is required (See ExperimentD).
(2 different stocks of Penicillin are referred to here. see p.84).

EXPERIMENT C

This experiment was arranged to test the resistance of Br. abortus to Crystal Violet in the

absence of tonsil tissue.

As we had now decided to attempt isolation of Influenzoid organisms from tonsils, we tested the resistance of these also against Crystal Violet in this experiment.(also in absence of tonsil tissue). Although this strictly belongs to Section II. of our thesis, we include it here since we actually combined the two in one experiment for comparison.

Four concentrations of Crystal Violet in broth were used, namely:-

c = 1/10,000 Crystal Violet
d = 1/20,000 " "
e = 1/40,000 " "
f = 1/80,000 " "

Fildes blood digest was added to every broth tube to make a concentration of 2%. This was done to supply "X" & "V" factors for the Influenzoid organisms, but was also included where only Br. abortus was being tested.

Cultures Used.

1. Two strains of Br. abortus were used - namely Abortus No. 16 and Abortus "Weybridge". A 24 hours culture on special medium tube (5" x 5/8") was washed off with 1cc broth in each case; and one loopful of each inoculated into a series of Crystal Violet broth tubes of above noted concentrations (c,d,e,f).

2. Five Influenzoid strains, isolated from our tonsils and chosen at random and 2 strains received from Prof. McLeod, of Leeds - used as controls, - were employed. A 24 hours culture on Fildes agar tube (5" x 5/8") was washed off in 1cc broth (containing 2% Fildes) in each case. From 1 to 4 loopfuls was the dose inoculated into the series of Crystal Violet broths - the dose being varied to see if there was any difference in resistance noted. The strains used were Nos. 27, 48, 64,

74, & 92 from Tonsils, and "Cooper" & "Coates" Strains as controls.

Into one series of Crystal Violet broth tubes, both Br. abortus and an Influenzoid were inoculated - to compare results with tubes containing only one or other of these. The "Weybridge" Strain of Abortus, and No. "48" Influenzoid were used for this purpose.

As both these strains of Br. abortus could grow in air, the whole experiment was carried out in ordinary atmosphere - at 37°C.

Subcultures were made from every tube (1) after 1 day. and (2) after 5 days incubation. These were made on to Ordinary agar (tubes) in the Br. abortus series, and on to Fildes agar tubes in the Influenzoid series, whilst those inoculated with both organisms were subcultured on to both Ordinary and Fildes agar tubes.

The results are recorded in Tables VIto IX. Subcultures from Influenzoids were noted after 24 hours (they always showed some growth within this time, if at all) - whilst those from all tubes containing Br. abortus were examined daily for 2 to 4 days.

Identification of organisms isolated.

(1) Br. abortus was identified by its cultural and microscopic appearance, and the fact that it usually required at least 48 hours to show any growth on Ordinary agar, but its identification was established

in every case (from every tube) by a complete agglutination test against abortus antiserum (1/50 to 1/3,200 dilutions).

(2) The Influenzoids were identified by (a) showing growth within 24 hours (b) naked-eye appearance of growth - small colourless colonies, and (c) microscopic examination of growth - tiny pleomorphic Gram negative bacilli. (d) growth occurring on Fildes agar and not on Ordinary agar. Where both Br. abortus and Influenzoids might be present this test 2(d) was applied - An example of this is seen in the Table IX "f" (after 5 days in Crystal Violet).

Conclusions from Experiment C.

1. Br. abortus is much more resistant to Crystal Violet than are the bacilli of the Influenza group. It survives 5 days in 1/10,000 concentration - in the absence of tonsil tissue. The result in Table IX suggests that it actually grows in this Crystal Violet broth, as it was isolated after 5 days incubation, but not after 24 hours (from 1/10,000 Crystal Violet).

2. The Influenzoid organisms (all true Haemoglobinophils) vary slightly in resistance to Crystal Violet; some survive 1/80,000 for 5 days, and some survive 1/40,000 for 5 days. None survived the 1/10,000, or 1/20,000 concentrations, even for 24 hours. The controls seemed to be somewhat more sensitive - one not surviving at all, the other surviving only the weakest Crystal Violet concentrations used (1/80,000).

The varied dosage of inoculum (Influenzoid) did not appear to make any difference. All the strains seemed to be definitely sensitive to Crystal Violet.

Continued page 84.

TABLES VI - IX.

Experiment with Br. abortus & B. influenzae in Crystal Violet.

Subcultures to Ordinary Agar & Fildes Agar (1) after 1 day
(2) after 5 days in Crystal Violet

TABLE VI.

Br. abortus.(Ordinary agar slopes) 2-4 days incubation.

		after 1 day(in C.V.)	after 5 days. (in C.V.)
Abortus "16"	c	++	++
	d	++	++
	e	++	++
	f	++	++
Abortus "Weybridge"	c	++	++
	d	++	++
	e	++	++
	f	++	++

TABLE VII.
B. influenzae (controls).
Fildes agar slopes - after 24 hours incubation.

		after 1 day(in C.V.)	after 5 days.(in C.V.)
"Cooper"	c	—	—
	d	—	—
2loopfuls	e	—	—
	f	+	+
"Coates"	c	—	—
	d	—	—
2 loopfuls	e	—	—
	f	—	—

++ indicates copious growth
+ " fair "
— " no "

TABLE VIII.

B. influenzae (Tonsil strains) 24 hours growth. (Fildes Agar)

		after 1 day (in C.V.)	after 5 days (in C.V.)
"27" 1 loopful	c	—	—
	d	—	—
	e	—	—
	f	#	+
"48" 2 loopfuls	c	—	—
	d	—	—
	e	—	—
	f	—	+
"64" 3 loopfuls	c	—	—
	d	—	—
	e	—	—
	f	+	—
"74" 2 loopfuls	c	—	—
	d	—	—
	e	+	+
	f	+	+
"92" 4 loopfuls	c	—	—
	d	—	—
	e	—	+
	f	+	+

\# indicates copious growth.

\+ " fair "

— " no "

The dose of initial inoculum (in loopfuls) is indicated for each strain.

TABLE IX.

Br. abortus & B. influenzae together.

after 1 day in Crystal Violet

Influenza"48" & Abortus "Weybridge"	Fildes Agars growth after 24 hours	Fildes Agars growth after 48 hours	Ordinary Agars. growth after 24 hours	Ordinary Agars. growth after 48 hours.
c	—	—	—	—
d	—	+	—	+
e	—	+	—	+
f	—	+	—	+

after 5 days in Crystal Violet

	Fildes Agars growth after 24 hours	Fildes Agars growth after 48 hours	Ordinary Agars. growth after 24 hours	Ordinary Agars. growth after 48 hours.
c	—	+	—	+
d	—	+	—	+
e	—	+	—	+
f	+	+	—	+

N.B. + = growth present — = no growth

\# = profuse growth.

c, d, e, f, = 4 concentrations of Crystal Violet viz:

1/10,000, 1/20,000, 1/40,000, and 1/80,000.

Tables VI - IX all give results of subculture from Crystal Violet.

3. It is interesting to compare these results (for Influenzoids) with those of Table 1. for Tonsils 25 to 30 in Section II. (p 121). In the latter, Influenzoids are recovered from 4 out of 6 tonsils, from much stronger Crystal Violet concentrations - namely, 1/7,500, and 1/10,000 - that is in presence of tonsil tissue (0.2cc). This shows the great difference that presence or absence of tonsil tissue makes in the case of the survival of Influenzoids in Crystal Violet.

4. Br. abortus, by contrast shows great resistance to Crystal Violet even in absence of tonsil tissue. We may conclude therefore that when tonsil tissue is present - as in our routine technique - its chance of survival should be still greater. This confirms conclusion 3 of Experiments B1. & B2.

II. Resistance of Br. abortus to Penicillin

Experiment D (Qualitative)

The object of this experiment was to test further the resistance of Br. abortus to Penicillin, using a more extended series of concentrations than those used in Experiment B2. This experiment is only qualitative. Here we employed the following series of Penicillin in broth - 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, & 1/1,280, - also a control tube, (broth only).

This was a fresh stock of Penicillin (7 days culture) of standard strength - and therefore stronger than that used in Experiment B2.(which was 14 days culture).

Three tonsil filtrates were used, Nos. 28,29, and 30, and to each was added emulsion of Br. abortus, and also of Staphylococcus. The latter was added so that we might at the same time test the effect of the

Penicillin upon Gram positive organisms, when the latter were in abundance.

Three series of Penicillin broth tubes were thus inoculated with the 3 mixtures - one to each - (Tonsil filtrate + Br. abortus & Staphylococcus) a loopful being used.

These were incubated at 37°C in air, (a strain of Abortus capable of growing in air being used).

Subcultures were made on the 1st, 3rd, 6th, and 8th days on to Ordinary Agar plates.

These were examined both naked-eye and microscopically for evidence of presence of Br. abortus. We did not succeed in isolating Br. abortus from any of these concentrations of Penicillin. The 3 strongest concentrations e.g. yielded the following:-

Penicillin 1 in 10	Gram + bacilli	Diphtheroid
" 1 in 20	" " "	
" 1 in 40	Gram — cocci	
Control (broth only)	Gram — cocci & Gram + cocci	

The weaker concentrations gave for the most part Gram positive organisms, Staphylococci etc.

The mode of preparation and uses of Penicillin are discussed under Section II..

Conclusions from Experiment D.

1. We may conclude from this experiment that Br. abortus is sensitive to Penicillin, in dilutions adequate to inhibit Gram positive organisms. (This is in presence of a small amount of tonsil tissue).

2. Penicillin in the stronger concentrations appears

to be useful for inhibiting Gram positive organisms, especially cocci. (1 in 10 to 1 in 40 strengths). - Tonsil tissue being present, the Gram positive cocci in this, as well as those artificially inoculated, (Staphylococci) are inhibited by these concentrations.

General Conclusion from Subsidary Experiments A, B, C,& D.

The most important practical conclusion to be drawn from these experiments is with reference to the question of the best technique for the attempted isolation of Br. abortus from tonsils. The problem was to find an agent which would effectively inhibit the Gram positive flora so abundant in the tonsils, but which would not prevent the survival or growth of Br. abortus if present - even in minimal amount. We have seen that this condition is fulfilled to a very great extent by the dye Crystal Violet. Penicillin on the other hand was not satisfactory for this purpose, as Br. abortus was not sufficiently resistant to it - but this agent is useful for inhibiting Gram positive organisms. We concluded therefore from these experiments that our best plan was to culture the tonsil tissue in Crystal Violet broth followed by subculture on Ordinary Agar; then if Gram positive organisms were present a second subculture to be made on Penicillin agar and a final subculture on Ordinary agar.

These Experiments therefore confirmed our adoption of the technique outlined in more detail on

page 65- which was employed throughout the rest of our Br. abortus investigations. This in brief, was the culture method evolved.

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SECTION 11.

To determine the incidence of Bacillus influenzae (group) in Extirpated Tonsils.

Part I. Review of the Literature dealing with Bacillus Influenzae (Pfeiffer).

Historical.

The disease Influenza is not a new phenomenon but has been recognised as a recurring visitor of this and many other countries for several centuries, at times being almost universal in its distribution. It was first definitely heard of in 1173, and the first pandemic recorded in 1510. Outbreaks occurred at irregular intervals varying from a few years to a few decades, some being local or epidemic, and others pandemic, in distribution. Many theories were invented to explain its occurrence, some connected with the weather (pestilential winds etc.), others with volcanic eruptions and earthquakes, and some even with political events (such as the Union of the Parliaments in 1707). Jack (1919).

Its contagiousness was suspected and investigated during the latter half of the 18th century, but though there was much evidence for this, it was not accepted by many.

The greatest pandemic of the 19th century was that of 1889-1890 which occurred after the birth of Bacteriology as a science. Attempts to isolate a causal organism were unsuccessful till Pfeiffer in Dresden isolated his bacillus during a smaller epidemic in 1892. The infective role had been

assigned to various organisms prior to this, such as Streptococci, Pneumococci etc. Pfeiffer's organism grew only on media containing blood, and this may account for previous failures to find it. The contagiousness of Influenza was generally accepted from this time. Pfeiffer examined only serious cases (with Pneumonia etc.) and found the organism in a large majority in sputum and also in post mortem material. It was a tiny, pleomorphic, Gram negative bacillus requiring blood in artificial cultivation, non-motile and non-spore bearing. There seemed to be good evidence of its relationship to Influenza and it was accordingly called Bacillus influenzae.

The symptoms and complications were described at various times and correspond with those we are familiar with since the 1918-19 pandemic. It was primarily an affection of the respiratory system though in some outbreaks (at least in some localities) catarrhal symptoms were absent.

It was known under different names at different periods, but our name Influenza appears to be derived from the Italian writers of the 16th century, who associated the disease with "una influenza di freddo"-- an influence of cold. From this the term "Influenza" first passed into English in 1743, in the writings of Pringle.

The term "Spanish Flu'" applied to the 1918-19 outbreak (because it first appeared in

Spain) was not new either, for "Spanish catarrhs " are recorded as far back as the 16th century. (Jack 1919.)

Although at first accepted by many as the causal organism of Influenza doubts have been frequently expressed as to the relation between the bacillus of Pfeiffer and Epidemic Influenza. We will omit the period between 1892 and 1918, when little real progress was made, due largely to lack of a satisfactory medium for the culture of this group of organisms. In 1918 a revolution in culture methods took place, when media containing blood changed in various ways and giving a luxuriant growth, were substituted for those containing unchanged blood, which gave only pin point colonies. (Fleming and MacLean 1930.) This greatly facilitated the isolation of B. influenzae and allied organisms. Previous to this Ordinary Blood Agar was used either (1) Agar smeared with blood (human or animal) or (2) Agar mixed with about 10% blood.

Cultural and Nutritional.

(a) Culture Media.

The first of these improved media was introduced by Matthews (1918), who used trypsin digest of blood (in Agar). It took 4 to 7 days to prepare, however, but in 12 attempts he isolated B. influenzae every time.

Levinthal (1918) at the same time commenced

using boiled and filtered blood with excellent results.

Fleming(1919) substituted blood broken down by strong mineral acids, and then neutralised, whilst Fildes (1920) used blood digested by pepsin with equally good results. This was more quickly prepared than by trypsin method.

Avery (1918) also introduced a useful medium --Oleate Haemoglobin Agar.

(b) Growth Factors.

Altered blood (or haemoglobin) is present in all these media. This peculiar dependence of the B. influenzae upon blood for growth aroused interest and was investigated by several observers. Davis (1917) appears to have been the first to point out that two factors are present in the blood, one heat stable, the other heat labile, and that both together are essential for growth of B. influenzae -- neither alone sufficing. This important observation was confirmed by Fildes (1921), Thjötta and Avery (1921) and Rivers (1921). The heat stable substance has since been called the "X" factor, and the heat labile the "V" factor.

It was believed for a time that this organism could not grow in the absence of blood pigment (Fildes 1921). It was thought that unaltered haemoglobin by its affinity for oxygen hindered growth of the organism, but haemoglobin altered by heat etc. lost this oxygen-affinity, and promoted growth of the bacillus by aiding transfer of oxygen

to it--its action being probably catalytic, (first suggested by Davis, then by Fildes (1921) and others.).

Thjötta and Avery (1921) studying the distribution of these growth accessory factors in blood concluded that both X and V factors are contained in the cellular fraction of the blood. They also showed that the V factor is present in yeast and vegetable cells, e.g. fresh tomatoes, green peas and beans, and concluded that it was a vitamine-like substance. The term "haemoglobinophilic" has been applied to this and allied organisms, and is more accurate than "haemophilic" which is also frequently used.

Rivers and Poole (1921) confirmed these findings, but stated that the X factor had not so far been found outside the blood. The X factor resists autoclaving 30 to 45 minutes under 15 pounds pressure, but this destroys the V factor, as does also prolonged boiling.

What is called the "symbiosis" phenomenon had previously been observed by several workers. When growing on ordinary blood agar along with other bacteria, it was noticed that B. influenzae grew more luxuriantly where it was in close proximity to such bacteria, e.g. Staphylococci. This was now explained by the fact that these other organisms when growing elaborate V factor, which diffuses in the medium and is used by the B. influenzae thus stimulating growth. X factor is sufficient in minute amounts.

Davis (1921) pointed out that strains of

B. influenzae which had been kept for years, as well as strains isolated during the recent pandemic, all required these 2 factors for growth.

Thjötta and Avery (1921) in searching for X substance outside blood, discovered that sterile raw potato supports luxuriant growth of B. influenzae and must therefore contain both X and V. Potato was known to contain peroxidases and catalysts. Banana was found to behave likewise.

They concluded that B. influenzae is not an obligatory haemophil, but that the growth accessory substances found in blood also exist in plant tissues.

Rivers (1921) confirmed this by growing B. influenzae on a medium containing fresh eggs (autoclaved 15 minutes), 2% peptone water, and filter-sterilized Yeast Extract. He grew it also on a similar medium substituting various chemical salts for the peptone, no blood derivative being present.

The terms Haemophilic and Haemoglobinophilic have, however, been retained and as work proceeded it became evident that there was a group of these organisms and that they could be classified according to their dependence upon one or both of these food factors. We have used the term "Influenzoid" in this thesis to denote organisms which culturally and morphologically resemble B. influenzae, calling those "True Influenzoids" which are dependent for growth upon one or both factors.

(c) Classification of Influenza group bacilli.

In 1903 Friedberger recovered from the preputial secretion of dogs an organism which resembled B. influenzae morphologically and in its dependence on blood for culture. It was called Bacillus haemoglobinophilus canis.

Rivers (1922) tested this organism's growth requirements and showed that it demands only the X factor. In the same year he isolated from 2 cases of Influenza an organism which was in every respect identical with B. influenzae, except that it required only the V factor for growth. He gave it the name Bacillus para-influenzae.

Fildes (1923) examined the growth requirements of B. influenzae, Koch-Weeks bacillus, B. haemoglobinophilus canis, and other bacilli. He found the first two identical, requiring both X and V factors, and the third required only X (confirming Rivers). He considers that only these 3 organisms should be included in the Haemoglobinophilic group and that others described as "Haemophilic" e.g. B. pertussis etc. should be excluded.

B. influenzae from the time of its discovery was generally recognised as non-haemolytic (when grown on medium containing fresh rabbit blood corpuscles). Pritchett and Stillman (1919) described an identical organism which was haemolytic and called it Bacillus "X", putting it in a separate group. Rivers and

Leuschner (1921) regarded it as a haemolytic variety of the Influenza bacillus and as belonging to that group. But it was Fildes (1924) who first proved that these haemolytic strains demand only the V factor for growth, being indifferent to the X factor. In this respect they are like B. para-influenzae which, however, is non-haemolytic. Fildes worked with 14 Danish strains received from Kristensen, who also (1922) refers to them as being less haemoglobinophilic than Pfeiffer's bacillus.

The following then are included in what Fildes (1924) calls the "Influenza group bacilli":

B. influenzae, requiring both X and V factors.
B. haemoglobinophilus canis, requiring only X factor.
Haemolytic Influenza bacilli, " " V "
B. para-influenzae (Rivers), " " V "
but non-haemolytic.

The Isolation of B. influenzae from Mixed Cultures.

(a) Use of Penicillin.

Probably the best method for accomplishing this is by the use of Penicillin as first recommended by Fleming (1929). The chief problem is to get rid of Gram positive flora, so abundant in respiratory exudates etc. Penicillin is an antibacterial substance produced by a mould, Penicillium notatum, when grown in broth and filtered. This substance is strongly inhibitory to Gram positive organisms

generally, especially the pyogenic cocci, whilst many bacteria, especially Gram negative ones, such as B. influenzae, are markedly insensitive to it. Fleming has demonstrated its usefulness in the isolation of B. influenzae. It can be used in 3 ways, (1) by spreading a few drops over an inoculated plate, (2) by adding it to fluid medium (broth) in suitable amount, e.g. 1 in 10 or 1 in 20, and (3) by incorporating it in a solid medium, in proportions required.

Methods (2) and (3) are most useful, the media being inoculated after Penicillin is added. We have made use of both these methods.

(b) Use of Tellurite.

Fleming (1932) also showed that Potassium Tellurite is useful in differentiating B. para-influenzae from true B. influenzae -- the former being more sensitive to this reagent. He showed that organisms which are Penicillin sensitive are usually Tellurite insensitive, and vice versa. He used both these agents together in this instance. We have also carried out tests with Tellurite.

Colonial Characters & Morphology.

B. influenzae has been described as a tiny pleomorphic Gram negative bacillus, non-motile and non-spore bearing. Its usual appearance is coccobacillary (typical), but filamentous forms have been described some with swellings and branches (atypical), and inter-

mediate forms occur. Kristensen (1922) gives a detailed account of the variations in morphology and colonial characters. He describes large and small colonies, rough and smooth colonies, and colonies which were darker in colour that others, all being transparent or translucent. Typical ones give smooth colonies, and atypical ones rough colonies, but the dividing line is not sharp. M.M.Smith (1931) showed that typical could be changed into atypical, and vice versa. Change in morphology and colonial formation occurred together. Pittman(1931) imvestigated these S.and R. strains (smooth and rough). S. strains produce smooth large colonies and tha individual bacteria are capsulated, whilst R. strains produce rough small colonies, of irregular outline, and the bacteria possess no capsule.

It seems to be generally agreed, however, that no satisfactory grouping of the Influenza bacilli can be accomplished on the basis of colonial characters and morophology.

General and Statistical.

The literature on the subject of Influenza is somewhat confusing and difficult to classify. There are, broadly speaking, 2 schools of bacteriologists (1) those who regard B. influenzae (Pfeiffer) as the primary etiological agent in Epidemic Influenza, and (2) those who deny this relationship, though admitting it is a frequent secondary invader.

Some of this school believe that a Filter-passing virus is the causal agent.

We will examine some of the findings of each school, dealing here only with cases of Influenza. Later the occurrence of B. influenzae in healthy persons will be considered.

First School -- Findings in Influenza Cases.

McIntosh (1918) examining throat swabs, sputum, and P.M. material from Influenza patients found 42 positive out of 56 = 75%. Wilson and Steer (1919) isolated the organism from 20 out of 24 sputa, from 57 of 72 bronchial swabs (P.M.) and from 12 of 71 heart blood cultures. They concluded that B. influenzae acts as a pioneer, and paves the way for other invaders, pneumococci, streptococci, etc. They compare it to the similar effect of poison gas. Roos (1919) found the bacillus in 50--90% of cases, and asserts that it can be found in every case of true Influenza if proper medium is used (altered blood). Spooner, Scott, and Heath (1919) recovered the organism from lungs (P.M.) in 62%, and in pure culture in at least 50%. Fildes and McIntosh (1920-21) give a review of the literature and put up a strong case for the etiological role of B. influenzae in Epidemic Influenza. They give numerous statistics, the highest being those of Schorer who found 91.5% of uncomplicated cases positive, and 93.8% of complicated ones positive. Medalia had the largest series, 2279

cases with 76.8% positive. In P.M. material McIntosh had 94%, and Keegan 82% positive. Many observers, they state, failed through using unsuitable media. McLeod, Ritchie, and Dottridge (1920-21) make a survey of the incidence of B. influenzae infections before, during, and after the 1918 pandemic quoting figures from numerous reports, as well as their own findings. They conclude that there was a low incidence of such infections (? due to faulty technique) before the 1918 outbreak, rising to a maximum during this pandemic, and falling away again after it had passed off. They found a parallel rise in the throats of healthy persons during same period. They think there is good evidence for regarding this organism as the cause of Influenza.

Little (1918-19) and Hopkins (1918-19) (discussion) report similar positive findings, and the latter concludes that the primary infection is due either to the B. influenzae, or to a Filter-passing virus. Carnegie Dickson (same discussion) supports Pfeiffer's bacillus, having found it in almost 100% of cases, although he found it also frequently present in cases of chronic bronchitis. Eyre (1918-19) is one of the strongest advocates of this school and states " I consider that B. influenzae completely fulfils the conditions required of an organism accused of specificity i.e. with the exception of the comparatively rare "carrier" it is absent from the upper air passages of normal individuals, is capable

of artificial cultivation, and such cultures are capable of initiating the desease in man, with recovery of the organism from the induced lesions".

Second School -- Findings in Influenza cases and in Certain Infectious Diseases.

Davis (1919) examined throat swabs from patients suffering from the following Infectious Diseases and found B. influenzae present as follows:-

Whooping Cough (68 cases)	89% positive
Measles	56% "
Varicella	64% "
Lobar Pneumonia	33% "

He concludes that there are as good reasons for saying that B. influenzae is the cause of Whooping Cough or Measles as for saying it is the cause of Influenza.

A diplococcus (or Diplostreptococcus) was frequently found in Influenza cases, especially early in the epidemic, and Davis considers there are as good reasons for regarding this as the cause of the disease, as for saying it is B. influenzae. He says that the Germans and Swiss lay more stress on this Diplostreptococcus, whilst the English lay more stress upon Pfeiffer's bacillus.

Lord, Scott, and Nye (1919) quote reports on the occurrence of B. influenzae in various infectious diseases e.g. in Tuberculosis cases 33% (children) (Wollstein), in Scarlet Fever cases 39% (Jehle), and in Diphtheria cases 60% (Jehle), and others which support Davis' findings (above). They conclude that it

is a common invader of the normal respiratory tract, and may be found in a considerable proportion of the contagious diseases of childhood. They regard it as a secondary invader only, in Influenza. Howard (1919) found it only once in 128 examinations (throat swabs etc.) of Influenza cases.

German writers seem to be mostly of this school, e.g. quoting from Abstracts (1918 J.A.M.A.) we find Schmorl isolated the Pfeiffer bacillus in only 3 out of 50 Influenza cases. Bernhardt and Meyer failed to find it at all in 28 cases (P.M.). Bernhardt in a previous series (47 cases) found a Gram positive diplococcus in the majority. Mandelbaum failed to find it in 31 cases, and he denies the relationship of the bacillus to Influenza. Selter had negative results in 33 cases, whilst Grüber and Schädel found it only in 14 of 250 cases examined.

We may also here mention Beaumont (1919) who from his own investigations, regarded Influenza as a Mycotic infection of the respiratory (and sometimes alimentary) tract.

These observers (and many others) all agreed in regarding Pfeiffer's bacillus as, at best, only a secondary invader, like Pneumococci and Streptococci.

We shall now review some experimental work dealing with

The Pathogenicity of B. influenzae for Animals and Man.

(a) Pathogenicity for Animals.

The laboratory animals used have included

rabbits, monkeys, guineapigs, white mice, and more recently, ferrets. It has been found, generally speaking, difficult to infect animals with B. influenzae. Results have varied greatly. Where symptoms or lesions have been produced it has been almost impossible to identify these with the symptoms and lesions seen in human Influenza, for these are quite protean and difficult to define. White mice have apparently been the most susceptible. We will deal chiefly with these as we used them also.

Jacobson (1901) using white mice, found that whilst they readily resisted B. influenzae in pure culture they succumbed if a sublethal dose of Streptococci (dead or alive) was inoculated along with the B. influenzae. The bacillus could be cultivated from the heart blood and viscera, showing that a fatal septicaemia had been produced. By passage through a series of mice (inoculated along with Streptococci) he found that the virulence of B. influenzae could be raised until it could produce a fatal septicaemia in pure culture. In such cases it was recoverable from the heart blood.

Kamen (1901), Roos (1916), and Wolf (1920) confirmed these results. The intraperitoneal method was generally used. The last named found that Influenza bacilli alone produced a serous peritonitis from which the mice recovered. The bacillus could not be found in the blood. The dose given was usually one slant, 24 hours culture. Pneumococci could be

substituted for Streptococci with similar results.

The general conclusion from these experiments was that the B. influenzae was dependent to a great extent upon the presence of certain companion organisms for the manifestation of its pathogenic qualities.

Other observers, however, found that pure cultures of B. influenzae ware lethal for white mice. Thus Wollstein (1911), Albert and Kelman (1919), and Ferry and Houghton (1919) whose results were confirmed and quoted by Hudson (1922) and (1924). The latter used strains from normal throats, common colds, and Influenza cases, all with lethal effect. The dose was a 22-24 hours culture on chocolate agar in tube 6" x 5/8" suspended in 1 cc saline, and injected intraperitoneally.

Hudson also found that these B. influenzae strains did not lose in virulence by being kept in subculture over several weeks. Most other observers had reported that there was a marked loss of virulence even after first subculture (Gundel and Linden 1930).

Hudson recovered Pfeiffer's bacillus in pure culture from heart blood in 68% of mice which died, and either pure culture or mixed culture in 86%. He states that B. influenzae invades the blood stream of the mouse even in sublethal doses, and multiplies under these conditions; but that its power to do this is enhanced when Gram positive cocci

are inoculated along with it.

The analogy between these findings and the mixed infections seen in Influenza cases (especially with complications) seems fairly obvious. Hudson also found that sublethal doses of B. influenzae conferred immunity to white mice against lethal doses.

Rosher (1931) pointed out that the inclusion of a small quantity of the X and V factors (Fildes blood digest) in the inoculum definitely increases the virulence of Influenza bacilli for mice,-- at least the indol positive strains of bacilli. The presence of these factors presumably stimulates multiplication of the organisms.

It seems pertinent to ask whether this factor has anything to do with the contradictory results of different observers. There does not seem to be any reference to this point in the technique of previous investigators. The inclusion of these factors in the inoculum might lead to positive results (death of the mice), where their omission would give a negative result. Much may sometimes hang upon a detail.

That a toxin is produced by B. influenzae in culture has been reported by several observers. Huntoon and Hannum (1919) prepared an endotoxin which had a toxic effect upon mice and guinea pigs, producing a congestion of the respiratory tract and haemorrhages into the alveoli and pleurae, which led to a predisposition to invasion by various organisms

with production of secondary lesions. Filtrates from cultures of Streptococci and Pneumococci did not produce such lesions, therefore apparently characteristic of B. influenzae. Parker (1919), Roos (1919), and Ferry and Houghton (1919) report similar findings. The last-named remark that the profound prostration produced in rabbits, due to a toxaemia, following injection of the toxin, closely simulates the early stages of Influenza in the human subject.

These different observers regard their results as evidence in favour of the possible role of B. influenzae as causal agent in Epidemic Influenza.

Blake (1920) inoculated 2 series of monkeys with B. influenzae and produced an acute infection of the respiratory tract, complicated by Pneumonia and Bronchitis in several of them. From the lungs (P.M.) of the Pneumonia cases he obtained B. influenzae in pure culture. He concludes that the symptoms and lesions are identical with Influenza in man, and supports the view that Pfeiffer's bacillus is probably the causal organism.

(b) Pathogenicity for man.

Several experiments have been done on human volunteers. They have been mostly unsuccessful.

The usual procedure was to instil (or spray) cultures of B. influenzae into the upper respiratory passages.

Rosenow (1918-19), McCoy and others, using mixed cultures, with few possible exceptions, had entirely negative results. Similarly with Yamanouchi et al., Lister and Taylor (1919) and Bloomfield (1920). At most, symptoms of a severe cold were produced, as in the isolated cases reported by Davis (1919) and Walker (1928).

In no case was a condition produced which could be diagnosed as Influenza.

Conclusions. Speaking generally we may conclude from these experiments in animals and man that B. influenzae can be pathogenic for both, but the evidence for its relationship to Epidemic Influenza remains inconclusive, although in certain animal experiments it is very suggestive.

Serological Investigations.

Several observers, including Fleming (1919) W.J.Wilson (1919), Spooner, Scott, and Heath (1919), and Edington (1920), reported presence of agglutinins to B. influenzae in Influenza cases. Wyard (1919) had negative results. Several recorded the presence of agglutinins in animals after repeated inoculation with B. influenzae, e.g. Spooner, Scott and Heath (1919)

Fleming and Cleminger (1919), Park (1919) and Valentine and Cooper (1919) examining numerous strains of influenza bacilli found them serologically very heterogeneous, "each being a law unto itself".

The evidence of Sharp and Jordan (1924), however, seems the most convincing. They found that

agglutinins to B. influenzae are present in even a larger proportion of Measles serums than of Influenza serums, and adduce evidence to prove that these agglutinins in both conditions are non-specific in character.

From this it may be concluded that ordinary agglutination reactions are of no practical value in Influenza. They are therefore not available as an aid to diagnosis, as for instance is the case in Undulant Fever or Typhoid (Widal Reaction).

Miscellaneous Findings.

A few of these merit attention.

Hoyle (1932) using mouse-inoculation studied cases of "Common Colds" and acute coryza. The more severe cases showed B. influenzae present in 80% to 95%. He concludes that the severity of an attack depends chiefly upon the presence or absence of this organism, (and pneumococci).

Russell and Fildes (1928) report a case of subacute infective endocarditis due to B. para-influenzae (of Rivers).

Taylor (1927) records a case of Pericarditis (in a boy of 6) due to B, influenzae. Other similar cases have been reported.

Somerford (1929) records a fulminating case of Septicaemia, in a child 2½ years, due to B. influenzae, (positive blood culture).

These examples are quoted as illustrating the fact that bacilli of the Influenza group can be definitely

pathogenic to man, whether they are the primary cause of Influenza or not.

A Filter-passing Virus.

Many bacteriologists support the view that Influenza is probably caused by a Filter-passing micro-organism.

The work of Bradford, Bashford and Wilson (1919) illustrates the pitfalls that may occur in this field of research. Their claims to have isolated such an organism (not only in Influenza, but also in Nephritis, Trench Fever etc.) had to be withdrawn after the criticism of Arkwright (1919).
Gibson, Bowman and Connor (1919) had similar findings in Influenza and claimed positive results with animal experiments, but these appear to be inconclusive (Logan 1919).

Olitsky and Gates (1920-21) claimed to have isolated such an organism from Influenza cases, and they called it Bacterium pneumosintes. Loewe and Zeman (1921), Gordon (1922), Olitsky and McCartney (1923), and others corroborated this finding, but McIntosh (1922) and Wilson (1927), with the same technique, had entirely negative results.

More recently Smith, Andrewes and Laidlaw (1933) working with ferrets, brought forward evidence for a Filter-passing virus as the cause of Influenza.

More investigations are required. The above illustrate the fact that findings and therefore conclusions by different observers are very conflicting.

Shope (1931) working on swine Influenza, has shown that this disease is only produced (experimentally) when B. influenzae (suis) and a filtrable virus (from cases of swine influenza) are combined, and not when either is used seperately. There may be an analogy between this and the human disease.

B. influenzae in Healthy Persons and in Children.

The literature on these subjects has a more direct bearing upon our own investigations.

(a) in healthy persons (adults).

The usual procedure was to examine naso-pharyngeal swabs. Some examinations were carried out during epidemics, and others during interepidemic periods.

One of the earliest reports is that of Pritchett and Stillman (1919) who found 42% positive for B. influenzae -- during an epidemic period. Lord, Scott and Nye (1919) examined throats of 34 young men and found 26 positive (=76%) -- also during epidemic. Dudley (1919) on board ship found 90% of throat swabs yielded Pfeiffer bacillus during the epidemic, but only 20-27% a month after it.

Bloomfield (1922) quotes statistics to show that before the 1918-19 pandemic the incidence of B. influenzae in healthy throats averaged about 20%; that this figure rose to 50% during the pandemic period, and then fell away again to the pre-epidemic

level (17%). He did not find any evidence of localisation of the bacillus in a diseased area or tissue, such as the Tonsil. Stillman (1922) examined 1077 normal throats during winters 1918-1921 and found 30% positive.

Fleming and McLean (1930) by use of Penicillin isolated B. influenzae from the gums of all of 30 healthy nurses and students, and from the tonsils and post-nasal spaces of nearly all of these. He also found B. para-influenzae in several of these. In a group of 6 laboratory workers he was able to isolate both B. influenzae and B. para-influenzae from the gums of all of them. One organism isolated required only X factor (like B. haemoglobinophilus canis). They conclude that members of this group of organisms are practically universally present in normal throats.

Kleineberger (1931) examined 622 healthy persons (children and adults) during 19 months following a local Influenza epidemic. He found 324 positive = 52%. The first 7 months (March-September 1929) gave an average of 58%, whilst during the following 12 months (October 1929-October 1930) it fell to 43.5 % -- showing a decrease in incidence as time went on.

(b) in children.

There are not many references in the literature to the incidence of B. influenzae in children; but we are considering them separately, as

our own investigations were exclusively in children.

N. Wall (1922) found this organism in 18% of extirpated Tonsils and Adenoids.-- This was in Liverpool when the city was free from epidemic. She concludes that "normally B. influenzae does not frequently inhabit the Tonsils and Adenoids of children".

Nakamura (1924) examined over 2000 Tonsils during 12 months and found 9% positive. Cobe (1930) out of 400 Tonsils found B. influenzae in 23%. Adults are included in this group. He noted a higher incidence during January-March than during September-December, and concludes that there is a seasonal variation in the incidence of this organism.

Kleineberger (1931) (in report quoted above) notes that in children the number of positive findings is very much higher than in adults, being 76% against 42% (in adults) in the first period, and 75% against 29% in the second.-- These figures are striking.

In the above reports the term "healthy" denotes that the persons were not suffering from Influenza or other obvious disease. It does not exclude "diseased tonsils" in those cases where these organs were examined.

Part II. THE PRESENT INVESTIGATION.

The Isolation of Influenza (group) Bacilli from Tonsils.

Introduction.

In Section I. when endeavouring to isolate Br. abortus from Tonsil Tissue we found that our purpose was best attained by the use of Crystal Violet to inhibit the Gram positive and other organisms, which are so abundant. During this investigation we noticed that tiny Gram negative bacilli resembling the Influenza bacillus (Pfeiffer) were frequently isolated by this technique, as is seen in Table III. (p. 62). In this experiment using 6 tonsils (Nos. 19 - 24) we found that these "Influenzoid" organisms were obtained from 4 of the 6 tonsils on Ordinary agar, and from 5 out of 6 on Fildes agar. On further subculture on Ordinary agar some of these died out which suggested they were Haemoglobinophilic, i.e. members of the Influenza group bacilli. Others survived repeated subculture on Ordinary agar, and were therefore not haemoglobinophil; these were examined as to the possibility of their being Br. abortus.

This observation suggested the desirability of examining our tonsil series not only for Br. abortus but also for these Influenza (group) bacilli - both being tiny Gram negative bacilli.

A preliminary experiment was therefore carried out to ascertain what technique was to be the

most suitable for this purpose.

During this investigation an outbreak of Influenza of mild form occurred in Dundee (and elsewhere). This lasted only about a month - during January 1933 - and then died away. We did not observe any fluctuation in incidence of the Influenzoid organisms from Tonsils before, during, or after this outbreak. Our investigation covered the period from beginning of December 1932 to end of March 1933.

Preliminary Experiment

It was decided to try Penicillin as recommended by Fleming (1929 & 1932), and as we were working with Crystal Violet we included it also to compare results, and to see which of these would best facilitate the isolation of this group of organisms from the tonsils.

We included Fildes broth in certain tubes and not in others to see if its presence made any difference (as supplying X & V factors required by B. influenzae); and as Fleming (1932) states that B. para-influenzae is more sensitive to Potassium Tellurite than is true B. influenzae, we tested this also in our preliminary experiment.

The experiment was as follows:- Six Tonsils were used Nos. 25 - 30, and they were inoculated into various concentrations of the following media - 0.2cc gauze filtrate was the inoculum in every instance.

To tubes containing each 5cc broth, the various ingredients were added. Fildes blood digest was added to make 2% strength.

The series was as follows:-

1. Ordinary broth + Penicillin (Pen. 1 in 2½ = A
   (Pen. 1 in 5 = B.

2. Fildes Broth + Penicillin (Pen. 1 in 2½ = C
   (Pen. 1 in 5 = D.

3. Fildes Broth + Tellurite 1/1,000,000 + Penicillin
   (Pen. 1 in 2½ = E
   (Pen 1 in 5 = F

4. Ordinary Broth + Crystal Violet (C.V. 1/7,500 = "b"
   (C.V. 1/10,000= "c"

For every tonsil we have thus a series of 8 tubes - A, B, C, D, E, F, & "b", & "c".

These tubes, inoculated with tonsil tissue, were incubated at 37°C. Those containing Fildes broth were subcultivated after 48 hours on to Fildes agar slopes. The remainder were subcultured after 6 days incubation. The resulting growths were examined not only naked-eye, but microscopically in every case, and the results are given in Table I.

The degrees of decolourisation of Crystal Violet broths (after 6 days) are given in Table II. and may be compared with Table II. of Section I.

TABLE I.

After 48 hours subcultured to Fildes Agar Slopes.
examined after 48 hours.

	C	D	E	F
		Tonsil No.		
25	Gram—cocci +++ Flu ++ Gram+cocci few	Gram—cocci +++ Flu + Gram+cocci few	Gram—cocci +++ Flu + Gram+cocci few	Gram—cocci +++ Flu + Gram+cocci few
26	Gram—cocci +++ Flu +	Gram—cocci +++ Flu ++ Gram+cocci+	Gram—cocci +++ Flu + Gram+cocci+	
27		Gram—cocci +++ Flu ++	Gram—cocci +++ Flu + Gram+cocci few	Gram—cocci ++ Flu ++ Gram+cocci few
28	Gram—cocci +++ Flu + Gram+cocci few	Gram—cocci +++ Flu ++ Gram+cocci few	Gram—cocci +++ Flu + Gram+cocci few	Gram—cocci +++ Flu ++ Gram+cocci few
29	Gram—cocci +++ Flu + Gram+cocci ++	Gram—cocci +++ Flu ++ Gram+cocci+	Gram—cocci +++ Flu ++ Gram+cocci few	Gram—cocci +++ Flu + Gram+cocci few
30	Gram—cocci +++ Flu +	Gram—cocci +++ Flu +	Gram—cocci +++ Flu +	Gram—cocci +++ Flu +

N.B. G—c = Gram — cocci
Flu = Influenzoids
G+c = Gram + cocci

26F & 27C were spoiled by accident

TABLE I (Continued)

After 6 days subcultured to Fildes Agar Slopes.
examined after 48 hours.

ex Penicillin		ex Crystal Violet	
A	B	"b"	"c"
Tonsil No.			
25			
Gram—cocci ++ Flu ++	Gram—cocci ++ Flu ++	Gram—cocci ++ Streps. +	Gram—cocci ++ Streps +
26			
Gram—cocci ++ Flu ++	Gram—cocci ++ Flu ++	Gram—cocci ++ Gram—bac + Gram+cocci few	Gram—cocci ++ Gram—bac + Gram+cocci few
27			
Gram—cocci + Flu ++	Gram—cocci ++ Flu ++	Gram—bac + Gram+cocci ++	Gram—bac + Gram+cocci ++ Streps
28			
Gram—cocci ++ Flu ++	Gram—cocci ++ Flu ++	Gram—bac + Gram+cocci ++	Gram—bac + Gram+cocci ++ Streps
29			
Gram—cocci +++ Flu +	Gram—cocci +++ Flu +	Gram—bac ++ Gram+cocci ++	Gram—bac ++ Tiny Gram+cocci ++ Streps.
30			
Gram—cocci ++ Flu ++	Gram—cocci ++ Flu ++	Gram—cocci +++	Gram—cocci +++

Flu = Gram —bacilli "Influenzoid"
Under C.V.Gram—bac (bacilli) also denotes "Influenzoid"

Gram+ = Gram positive
Gram— = " negative.

TABLE II.

Tonsil No.	"b" 1/7,500	"c" 1/10,000
25	##	+
26	##	##
27	—	—
28	+	##
29	—	+
30	—	+

— ## = complete decolourisation
+ = partial "
— = no "

In Table I. we see that Influenzoid organisms were cultivated from all of the 6 tonsils in A & B, also from C & D & E & F. The growth was rather more profuse from A & B. From the 2 Crystal Violets "b" & "c" only 4 of the 6 tonsils yielded Influenzoid organisms.

From these results we made the following deductions:-

(1) There is sufficient tonsil tissue to give the X & V factors required for true Influenzoids when Penicillin alone is used (A & B) therefore Fildes solution is unnecessary. Tellurite also confers no advantage; so that C, D, E, & F, are not required.

(2) Penicillin allows of Gram negative bacilli being recovered more often than does the Crystal Violet technique.

Other points may also be noted.

(3) In C, D, E, F, (subcultured after 48 hours) a few Gram positive cocci have survived in most cases. In A & B (which were exposed to Penicillin 6 days before subculture) no Gram positive cocci survived. The longer exposure to Penicillin in A&B would explain the difference.

(4) Comparing A & B with the Crystal Violet series "b" & "c" we note that Gram positive cocci have also survived in most of the latter (5 out of 6). This shows that Penicillin is more effective in inhibiting Gram positive organisms than is Crystal Violet, i.e. in concentrations which allow isolation of Influenzoids.

(5) We also note that Gram negative cocci are present in all the Penicillin series (A to F) - but less so in the Crystal Violet series. This is one of the chief disadvantages of using Penicillin. This problem is dealt with later.

We will now follow up more closely the subcultures made from A & B (Penicillin 1 in 2½ and 1 in 5). The original subcultures were on Fildes agar slopes. From these, single colourless Influenzoid colonies were picked out and subcultured to Fildes

Agar tubes - in an attempt to get pure cultures. In this way we were successful in procuring a pure culture of an Influenzoid organism from all 6 tonsils (25 to 30), although it was in some cases difficult owing to presence of Gram negative cocci (Flavus type). Each culture was examined microscopically, using Gram's stain, counterstaining with Carbol Fuchsin 1 in 10 - to confirm its Influenzoid character and pureness of culture. Tiny Gram negative (more or less pleomorphic) bacilli were found in every case - in pure culture.

Stock cultures of these were made and kept for further investigation.

The Preparation of Penicillin.

It has been demonstrated by Fleming (1929) that a certain mould Penicillium notatum produces in culture a powerful antibacterial substance, which is called Penicillin. The best medium for its production is ordinary broth. This substance reaches its maximum concentration in the broth in about 7 days at 20°C and after 10 days it steadily diminishes until it has almost disappeared after 4 weeks. It is readily filtrable. After 10 to 14 days at room temperature Penicillin loses most of its power, but can be preserved longer by neutralization. It is alkaline when freshly prepared. It resists boiling for a few minutes

but is practically destroyed by autoclaving for 20 minutes at 115°C. Speaking generally its antibacterial action is most marked on the pyogenic cocci, whilst the least sensitive organisms are the Gram negative bacilli. It appears to be non-toxic to animals. (Fleming).

In preparing our first lot of Penicillin the mould was allowed 14 days to grow (instead of 7) and consequently this preparation was less potent. Hence our use of it in such strong concentrations, (1/2½ & 1/5) in the above preliminary experiment. This was replaced by a fresh stock, prepared after 7 days incubation, filtered and neutralised. It was found that this kept well in the ice-chest and retained its potency after 3 months, and probably much longer. We tested this with our next batch of tonsils to find out what concentrations would be effective.

Experiment II.

Using fresh stock of Penicillin and Tonsils 31 - 36.

Penicillin was added to tubes containing broth (5cc) in concentrations 1 in 10 & 1 in 20 - 6 of each; and these were inoculated with 0.2cc gauze filtrate of each of the above 6 tonsils. After incubating 24 hours at 37°C subcultures were made from the upper part of these fluid cultures on to Fildes Agar (Slopes). These were examined after 24 hours incubation, and colourless transparent Influenzoid

colonies were present in all, with Gram negative cocci also present in greater or less degree. The 1 in 10 series were more satisfactory, so only these were further examined. Isolation of single colonies and subculture to Fildes Agar (tubes or plates - the latter where isolation was more difficult) was now carried out. In this way we were able to obtain pure cultures of Influenzoid bacilli from all these 6 tonsils. One or two were difficult, and required repeated attempts for success, owing to profuse growth of Gram negative (Flavoid) cocci.

The Penicillin-resistant Gram negative cocci (Flavus type)

These organisms form part of the normal basic flora of the tonsils. Our experiments with Penicillin showed that they were very insensitive to its presence. In our culture tubes containing Penicillin broth plus Tonsil tissue we noticed that a very fine yellowish grey pellicle or "Scum" formed on the surface of the broth. It was less marked and sometimes absent after 24 hours incubation, but almost invariably present after 48 hours. If kept longer it became more dense, and tended to grow slightly up the sides of the tube (meniscus like).

It was decided to make a Microscopic examination of (a) this Scum
and (b) the underlying culture, which was turbid.

Two cultures (Nos. 27 & 29) where isolation of Influenzoids was difficult, were chosen and the following were the findings.

(1) 27. (a) scum = Gram negative cocci only
(b) underlying fluid = Gram negative cocci and Gram negative bacilli

(2) 29 (a) scum = Gram negative cocci and a diphtheroid.
(b) underlying fluid = largely Gram negative bacilli.

The scum consisted therefore chiefly of Gram negative cocci whilst the subjacent fluid contained mostly small Gram negative bacilli. The problem was how to exclude or reduce the growth of these cocci.

We decided to test the effect of centrifuging; the idea being that as influenzoids are tiny while Flavus is large some separation in fluid medium might be attained by this procedure. Centrifuging was carried out for 20 minutes on second last button of ordinary centrifuge. Subcultures were made from the same tubes both before and after centrifuging - to compare resulting growths.

The result obtained was that enrichment of the Influenzoids as compared with the Flavus occurred (in the upper part of culture).

Two other simpler procedures, however, were found to give as satisfactory results, and so rendered centrifuging unnecessary.

These were as follows:-

(1) period of incubation - Subculturing within 20 -24 hours before scum in many cases had formed.

(2) Where scum was present at 24 hours by holding tube obliquely, the platinum loop could avoid it, and be inserted into the subjacent fluid.

also

(3) by plating and spreading - colonies could be well separated.

With this simplified technique, the results were very satisfactory although the Flavoid cocci could not be got rid of, and were in fact nearly always present on the plates, but separate colonies of Influenzoids were usually numerous and easy to isolate. In the majority of cases these 2 organisms showed approximately equal growth; in some the Gram negative cocci predominated, whilst in others the Influenzoids did so. In a small percentage the first subculture gave a pure culture of one or the other group of organisms.

It should be noted that we made our subcultures from the upper part of our fluid cultures (Penicillin broth). The tonsil tissue (after the first mixing) sank to the bottom of the tubes, and formed a deposit. There was apparently sufficient X & V factors distributed throughout the medium (from the tonsil tissue) to favour proliferation of the Influenzoids. Perhaps Penicillin, being of vegetable origin, may contain V factor, and may thus stimulate growth of these organisms. Throughout our work,

the impression was certainly formed that these Haemoglobmophilic organisms not only survive, but multiply and flourish in the presence of Penicillin.

Technique adopted.

We will now summarise the routine procedure which we discovered most satisfactory for the isolation of these Influenzoid bacilli from tonsils, and which we adopted throughout the whole of our investigation. The steps were as follows:-

(1) Inoculate Penicillin broth 1 in 10 with Measured amount of tonsil tissue (0.2cc tonsil filtrate); and incubate 24 hours at 37°C (in air).

(2) Subcultivate in 20 - 24 hours to Fildes agar plates - holding tube obliquely (to avoid scum when present) and withdrawing a small loopful of turbid culture just below surface.

(3) After 24 hours incubation isolate and subcultivate Influenza -like colonies to Fildes Agar tubes.

(4) After 24 hours incubation examine growth macroscopically and microscopically to verify pure culture of Influenzoid bacilli (Tiny Gram negative pleomorphic cocco - bacilli).

(5) Make Stock culture of all strains isolated for further investigation.

We will discuss the morphology and colonial characters of these organisms when dealing with various tests. Our methods of making stock cultures was as follows. Tubes containing semisolid agar (5cc — 10cc) were heated (standing in water) till melted; then withdrawn, allowed to cool a little,

and a few drops of blood (either our own, or rabbit's or guineapig's) added (rotated and stood in cold water to mix). These tubes were then heated to boiling point, lifted out of the water, and cooled. They were then ready for use. Each tube was inoculated with a separate strain of Influenzoid bacilli, The altered blood provides the necessary X & V factors, and cultures keep well for at least 10 days, after which they were always renewed.

The Testing of these Influenzoid Bacilli isolated from Tonsils.

As our investigations proceeded we accumulated a large number of strains of these organisms, as most tonsils yielded positive results. The question at once arose -- Were these true haemoglobinophilic bacilli (or as Fildes calls them "influenza group bacilli")?, and if so, the further question followed -- Were they the true Bacillus influenzae of Pfeiffer?.

The former question is easily settled by culturing on ordinary agar -- being careful not to carry over any of first medium (X and V factors) with the inoculum. True haemoglobinophilic organisms will not grow on this medium.

The second question is much more difficult to answer. It is generally agreed that we do not yet possess a satisfactory definition of what a true B. influenzae of Pfeiffer is. It is generally recognised

as having certain morphological, cultural, nutritional, and biological characteristics, which separate it from other organisms: but there is apparantly no means of "clinching the diagnosis" e.g. by animal inoculation, for its pathogenic effects are not constant in the same species of animal, and it cannot be identified by specific serological reactions. There is apparently a large number of strains, "each a law unto itself" serologically. In seeking for some test, which might be used to differentiate the true B. influenzae (Pfeiffer), we have at the present stage of our knowledge to be content with the combined results of several tests. Even these however seem to be of uncertain value as a means of identifying this rather complex organism (or group of organisms).

At the time of the 1918-19 Influenza pandemic B. influenzae was usually defined as a tiny haemophilic Gram negative bacillus, pleomorphic, non-motile, non-spore bearing. This definition was found to be too loose. Since then much research has been done, growth and other tests being applied, but a satisfactory definition has not yet been attained. An Influenza group of bacilli has emerged, but we are still seeking a means of differentiating the true Pfeiffer bacillus from the other members of the group.

We decided to examine our tonsil strains as thoroughly as possible, and accordingly submitted them all to the following series of tests which have been recommended by various observers. We include

here for the sake of completeness the ordinary tests of colonial characters and morphology.

The examinations were as follows:-

1. Colonial Characters.
2. Morphology.
3. Growth on Peptone Salt Agar + Fildes blood digest.
4. " " " " " (alone).
5. " " " " " + Yeast Extract.
6. " " " " " + Autoclaved Blood.
7. " " " " " + Yeast Extract + Autoclaved Blood.
8. " " Ordinary Agar + Fildes digest + Fresh Rabbit Blood.

for presence or absence of Haemolysis.

9. Oxidase Reaction.
10. Tellurite Tolerance Test.
11. Suspension in Saline -- whether easy or difficult (Hoyle).
12. Mouse Inoculation.

Rivers (1922) carried out a few other tests e.g. indol formation reduction of nitrates to nitrites, Amylase formation, and reaction in blood-broth-milk. The first of these has been applied by several observers, dividing strains into indol-positive and indol-negative -- but the others do not appear to have been so much used. We did not include any of these in our series.

1. Colonial Characters.

We have already noted some observations by others on this subject (page 101) which we need not repeat. In our series most of the colonies were colourless and transparent. Some, however, were translucent and of a slightly brownish tinge. Smaller and larger sizes occurred, and very occasionly giant

colonies were seen. Speaking generally the transparent colourless colonies were smaller in size, whilst translucent slightly brownish ones tended to be larger in size, though not invariably. At times -- the darker Influenzoid colonies, and the paler varieties of Flavoid colonies, were not unlike in appearance, though the latter were more opaque or opalescent in transmitted light. Occasionally a mixed colony was seen -- with opaque Flavoid in the centre, surrounded by a colourless Influenzoid ring. We did not see any rough or wrinkled colonies (mentioned by Kristensen, Fleming and others).

2. Morphology.

This is also referred to on page 101 . The organisms we isolated were in all instances tiny Gram negative bacilli. Most of the strains showed the characteristic pleomorphism quite plainly from the start. In some however it was not so evident at first, but became more definite in subsequent subculture. All strains were of the cocco-bacillary type. The average film showed short, stumpy, broad forms, ovoid or coccoid forms intermingled with rods of variable length and thickness. We did not meet any filamentous (atypical) or branched forms. Ours would appear to be all of the typical variety.

All strains stained quite well with Carbol Fuchsin, although a variegated effect was usually present -- some individual elements being almost purple or black, whilst others were pale pink ---

with all the intermediate shades present. Gram's method was always used with Carbol fuchsin 1 in 10 as counter stain. It was evident that some elements stained more darkly than others. This was observed by Kristensen (1922) who points out that these dark staining elements are not to be regarded as Gram positive, for staining with Carbol Fuchsin alone, or with certain other dyes, produces the same result. He regards them simply as elements with an excessive power of absorbing dyes. This variegation in staining occurred so constantly in our series that it seemed to be characteristic.

We did not find capsulated forms in a single instance. Bipolar staining was observed in some cases ~~after~~ after passage once through mice --in direct smears of heart blood -- but not apart from this.

Thus far, all our strains appear to belong to one group, indistinguishable colonially or morphologically.

Results.

With the Penicillin technique we were able to isolate from 89 tonsils (of our 100 series) organisms which, from their colonial and microscopic appearances, we judged might belong to Influenza group bacilli.

We were convinced however that these organisms were present in every tonsil, and that our failures were due either to faulty technique (plates

being too thickly spread) or to accident. Several plates showed only 1 or 2 Influenzoid colonies closely surrounded by Flavoids. Three other strains had been isolated but either died out or were scrapped (because contaminated).

We now proceeded to apply the various nutritional and other tests to these 89 strains to prove whether they were members of the Influenza group and to classify them.

Control Strains.

Through the courtesy of Prof.J.W.McLeod of Leeds, we received 5 Influenza strains which we used as controls, and which we submitted to all the various tests for comparison.

The designations of these strains were as follows:-

(1) "Cooper"strain. Typical Pfeiffer. Suspends easily in water and saline.

(2) "Coates" " " " Suspends easily in water and saline.

(3) "Mark" " Atypical. Difficult to suspend.

(4) "Noble" " " " " "

(5) "Wheatley" " " " " and Haemolytic.

Two strains are typical Pfeiffer, two atypical, and one atypical and haemolytic. The 3rd.strain "Mark" failed to grow so had to be omitted.

3. Nutritional Tests.

We will now consider the various nutritional

tests which were applied to these organisms. As our stock cultures accumulated we usually carried these out in batches of about 2 dozen at a time.

We have already seen that dependence upon certain food factors for growth (X and V) is the chief characteristic of the true B. influenzae; whilst others of the group are distinguished by requiring either the V factor only or the X factor only.

These 3 subgroups form the Influenza group bacilli. We accordingly made cultures of all our strains on the following media. (Subcultures were made from stock on to Fildes Agar tubes, and from these, after 24 hours on to these media).

We decided to use Peptone Salt Agar (P.S.A.) as the basis of all these media, as used by Rivers (1922) as this is reckoned to be free from minute quantities of X factor which may sometimes be present in Ordinary Agar. We began by using the latter, but found it gave appreciable (though delayed) growth in several instances where it was not expected. We suspected that one lot at least of this medium was supplying some accessory food factor, as one series of strains all showed growth. Both the egg used in its preparation, and the meat infusion, may contain X factor.

The Peptone Salt Agar contained:-
2% Agar + 2% Peptone + 0.5% Sod. Chloride.
The food factors were added to this as required.

Plates were used throughout.-- 3 or 4 strains

on each plate.

(a) Peptone Salt Agar + Fildes blood digest.(6%)

This is practically equivalent to Fildes Agar -- but contains a little more Fildes digest. We included this to complete the tests and to act as a control. Haemoglobinophilic organisms grow well upon this medium (both X and V present), and show the characteristic colonies already described.

(We compared growths in 24 hours with 2%, 4%, and 6% Fildes solution + P.S.A., and found the 6% much surpassed the others in richness of growth, so used this throughout).

Results.

All strains grew well upon this medium, forming transparent or translucent Influenza-like colonies.

(b) Peptone Salt Agar (alone).

Haemoglobinophilic organisms show no growth on this medium (X and V both absent). Many other organisms can grow on it.

Results.

Nine of our 89 strains showed definite growth on this medium, and were accordingly regarded as non-haemoglobinophil. (They showed growth on all the media used).

One other strain (No.12) gave a trace of growth on Peptone Salt Agar. It also gave a trace of

growth with V factor only, but none with X factor. We are inclined to regard the slight growth on P.S.A. as due probably to some X and V factor having been transferred with the inoculum. On the other hand Kristensen mentions that some strains are less strictly haemoglobinophil than others. We have included this strain in the Influenza group.

All the other 79 strains failed to grow on P.S.A. medium, but grew well on medium (a) above, and are therefore classed as true haemoglobinophils. Including No.12, we find therefore that 80% of our tonsils have yielded these Influenza group bacilli.

(c) Peptone Salt Agar + Yeast Extract (1 in 10).

Yeast Extract was used to supply the V factor as recommended by Thjotta and Avery,(1921), Fildes, (1924) and others. It was prepared by the Cold method, as used by Fildes (1924), and kept well in the ice-chest. We added it to the P.S.A. to give concentration 1 in 10.

This medium contains V factor, but not X.

Results.

Fourteen strains showed growth with V factor only. In three it was marked, in eleven it was a trace but definitely present. In nutrient requirements these correspond to the B. para-influenzae (Rivers,1922), which Fleming and McLean (1932) found almost universally present in normal mouths. Our strains were non-haemolytic. The "Wheatley" control, haemolytic strain

also grew on this medium--- Fildes (1924) found that Haemolytic strains require V factor only.

(d) Peptone Salt Agar + Autoclaved Blood.

Sheep's blood was used, and autoclaved for 30 minutes at 15 pounds pressure, then added to the P.S.A. The autoclaving destroys the V factor, but not the X.

Results.

Seven strains grew with X factor alone, five showing marked growth, and two just a trace. In food requirements these resemble B. haemoglobinophilus canis. Fleming (1930) found a similar organism in the mouth of one of his cases.

We may note here 3 strains (Nos. 72, 87, and 89) which grew on both V or X alone. Two gave fair growth and one (No.89) a trace -- with either factor. They failed to grow on P.S.A. Otherwise they behaved like true haemoglobinophils. Whether these are due to faulty technique, or are either irregular or "mixed" strains we do not know. Park (1919) refers to cases where more than one strain may grow from one parent colony. These may represent a mixture of 2 strains, one demanding X factor only, and the other V factor only -- and each showing growth in turn.

(e) Peptone Salt Agar + Yeast Extract (1in 10) + Autoclaved Blood.

This medium contains both X and V factors,

and therefore all strains should grow on it (as on (a) above).

Results.

All strains grew on this medium, as was to be expected, since it contained both nutrient factors.

Classification of our Tonsil Strains (Influenzoids).

If we deduct the strains which could grow with X or V factor only, we find there were 55 strains which showed complete dependence on the presence of both these factors. These behaved therefore like true influenza bacilli.

Our Influenzoid strains fall therefore into the following groups, based on good requirements.

Table 111.

(1)	Requiring X + V factors	55 strains.
(2)	" V only	14 "
(3)	" X "	7 "
(4)	Growing on X or V but not on P.S.A. (? mixed strains)	3 "
(5)	Showing trace of growth on P.S.A. ("doubtful" strain No. 12)	1 "
	Total	80 strains.

4. Haemolysis Test.

This was carried out to see if any of our strains were haemolytic. The medium used was Ordinary Agar + Fildes Solution (6%) + Fresh Rabbit Blood (5%). It was carried out along with the nutrient tests. The

strong concentration of Fildes solution was used to give strong growth, and allow results to be read in 24 hours. Ordinary Agar can be used here as it is not a nutritional test.

All the nutrient media were carefully inoculated to avoid carrying over X and V factors from original culture (Fildes Agar). We also avoided inoculating too liberally, to make the tests more fine. All media (Nutrition and Heamolysis) were examined after 24 hours inoculation at 37°C, and findings recorded.

Results.

This test was negative throughout, so that none of our strains were haemolytic. Only the control "Wheatley" strain was positive.

5. The Oxidase Reaction

This reaction is dependent upon the fact that certain organisms produce enzymes, known as oxidases or peroxidases, when grown on suitable media. These particular enzymes appear to act like oxidising agents, their function being to aid in the transfer of oxygen to the bacteria concerned. Their presence is tested for by the use of a chemical agent which is readily oxidised, this change being accompanied by a colour reaction.

This test was used by Hoyle (1932) who states that "a true influenza bacillus was considered to be a haemophilic non-haemolytic organism of predominant cocco-bacillary morphology, whose colonies on chocolate agar gave a weak oxidase reaction with tetramethyl paraphenylene-diamine hydrochloride"; and McLeod (private communication) mentions that this test is given with regularity by strains which, either from the circumstances of their isolation in man, or from the observed effect on the mouse, they judge to be pathogenic.

We carried out this test with our strains, growing them on "chocolate" agar and testing with the Tetramethyl compound (1% Solution). Two or three drops of this were applied by a pipette to the Culture and resulting colour change noted. The appearance of a blue colour indicates a positive reaction, but the colour varied with different strains from a pale blue

(weak positive) to a more marked blue (positive) and an intense blue (strongly positive). Absence of blue colour indicated a negative reaction.

The medium used was the same as that described on page 56 for growing Br. abortus except that Glucose was omitted.

All these oxidase tests were done after 20 to 24 hours culture (not longer) as reactions apparently vary with the age of the culture (McLeod). The test was carried out in 64 of the strains, and results are indicated in Table IV.

RESULTS.

Our findings were most variable. Strains identical in food requirements gave quite diverse reactions, some being negative, others strongly positive; others intermediate.

TABLE IV.

Sub-Group	Weak positive (weak+) strains	Positive (+) strains	Strongly positive (++) strains	Negative (−) strains
(1) Requiring X & V	8	5	7	21
(2) Requiring V only	2	2	1	7
(3) Requiring X only	nil	1	6	nil
(4) Growing on X or V	nil	nil	2	1
(5) "Doubtful" strain No. 12.	1	nil	nil	nil
Totals	11	8	16	29

It is seen that 35 strains gave a positive reaction (of varying degree), and 29 were negative. Of the 41 "true" influenza (X+V) strains 20 gave a positive and 21 a negative result.

Sometimes it seemed as though the degree of reaction varied directly with the amount of growth present.

According to McLeod (private communication) the true (pathogenic) Influenza bacillus gives a weak positive reaction; but he states that neither he nor Hoyle have followed the matter up so closely as to state dogmatically that there are strains which never give this reaction at all; and that even with the positive strains it varies with the age of the culture. If our 41 strains are "true" influenza then approximately 50% are oxidase-positive and 50% oxidase-negative. Perhaps this is an argument against their being the true Pfeiffer's Bacillus.

One other point which strikes us is the large proportion of the 3rd group (requiring X only) which give a strongly positive result (6 out of 7).

Amongst the Controls the 2 typical strains were positive whilst the Atypical ones were negative.

6. Tellurite Tolerance Test.

Fleming has found that Potassium Tellurite is

useful for distinguishing true B. influenzae from B. para-influenzae. The latter, he states, is more sensitive to it than is the former, being inhibited by a concentration of 1/1,000,000. We thought it might be useful to apply this test to our strains so carried out the following preliminary experiment. Plates were prepared containing Ordinary Agar + 3% Fildes to which Potassium Tellurite was added in 4 different concentrations as follows. (6 of each).

(a) Tellurite = 1 in 250,000
(b) " = 1 in 500,000
(c) " = 1 in 1,000,000
(d) " = 1 in 2,000,000
} in Fildes Agar.

Six of our Tonsil Influenzoid strains were chosen at random, 24 hours cultures prepared, and each strain was inoculated on to each of these 4 strengths of Tellurite. (24 tubes in all). The numbers of the strains used were 25, 27, 29, 32, 34, & 36. They were incubated at 37°C.

The tubes were examined after 24 hours, and all with one exception, showed not only growth, but good growth present. In 29 (a) the growth was feeble. They were examined microscopically and their Influenzoid appearance verified.

It was decided to repeat this Experiment using stronger concentrations of Tellurite as follows:-

(e) Tellurite = 1 in 25,000
(f) " = 1 in 50,000
(g) " = 1 in 100,000
(h) " = 1 in 200,000

In this series after 24 hours incubation, a strong growth was present in every tube, the only exception again being 29, this time in (e) & (f), where growth was feeble.

We again repeated the experiment using this time the 3 following Concentrations of Tellurite.

A Tellurite = 1 in 20,000
B " = 1 in 40,000
C " = 1 in 80,000

} in Fildes Agar (Plates)

These plates were (as before) carefully inoculated (not too liberally) with a larger number of Influenzoid strains (including those used above), and examined after 24 hours. Most of them showed no growth on "A" plates (1 in 20,000). Some showed growth on "B" plates (as well as on "C"). Others showed growth on "C" plates only and not on "A" or "B". It was interesting to note this differentiation amongst the strains in their resistance to Tellurite. Of the batch of 17 strains thus tested 1 strain grew on A plate (=1 in 20,000) as well as B & C = Tellurite Tolerance A; 7 strains grew on B plate (=1 in 40,000) (as well as C) = Tellurite Tolerance B; and lastly 9 strains grew on C plate only (=1 in 80,000) = Tellurite Tolerance C. All these were noted after 24 hours incubation. We always incubated them another 24 hours, and noted results to compare 24 & 48 hours growths. The latter always showed increase in growth and sometimes presence of growth where it was absent at 24 hours. But for our purpose of

differentiation amongst strains we found 1 day's growth more satisfactory.

RESULTS.

We give an analysis of our results in Table V. using the letters A, B, & C to indicate the 3 degrees of Tellurite Tolerance as described above. Only 3 of our series failed to grow on "C" concentration and these are indicated by the negative sign —.

TABLE V.

Sub-Group	Tolerance A Strains	Tolerance B Strains	Tolerance C Strains	Negative (=less than C) Strains
(1) Requiring X+V	23	14	15	3
(2) Requiring V only	6	4	4	nil
(3) Requiring X only	5	nil	2	nil
(4) Growing on XorV	2	1	nil	nil
(5) Doubtful strain No. 12.	1	nil	nil	nil
Totals	37	19	21	3

Tolerance A denotes resistance to 1 in 20,000 Tellurite
" B " " " 1 in 40,000 "
" C " " " 1 in 80,000 "
Negative " " " less than C (failure to grow in 1 - 80,000 Tellurite).

Nearly half the strains show marked tolerance (A), whilst the other half are equally divided between B & C. Only 3 show tolerance of less than 1 in 80,000.

It seems difficult, to draw any useful deductions from these findings; except perhaps that

this test is of no value in grouping these Influenzoid organisms, e,g, the 14 strains which demand only V factor (and thus resemble B. para-influenzae) - show as great tolerance for Telluriteas do the 55 strains which demand both factors (like true B. influenzae). This does not agree with Fleming's observation regarding Tellurite sensitiveness of the former organism.

We tried to correlate Oxidase Reaction and Tellurite Tolerance. We note e.g. that of the 16 strains giving a strongly positive Oxidase test, 12 show A, 2 show B, and 2 show C degrees of Tellurite Tolerance. Speaking generally, however, there seems to be no relationship between the two reactions.

Of the Controls the 2 typical strains show Tolerance A, as does also the haemolytic strain. The other Atypical strain ("Noble") shows Tolerance B.

7. Saline Suspension Test.

McLeod (private communication) mentions that Hoyle has been following up Fleming's work (using Penicillin) and that a considerable number of strains isolated by this method differ from the "true" influenza strains by the fact that they are not easily rubbed up to a smooth suspension, a character which is very marked in the case of Pfeiffer's bacillus. The suspension may be made with either water or saline. (see page 135 Controls).

We had not given attention to this point, but now decided to test all our strains in this way. The procedure was simple - A loopful of culture of each strain was mixed with 1 or 2cc of saline in a test tube, and it was noted whether they mixed readily, to form a uniform suspension. We used the controls for comparison, noting the contrast between the typical and atypical. The latter clumped together and sank readily to the bottom of the tube.

RESULTS.

Without exception, all our strains suspended easily and in this respect resembled the true Pfeiffer strains.

8. Mouse Pathogenicity Test.

We thought it desirable to carry out this test along with the others, as affording evidence as to the nature of these Influenzoid organisms. We used white mice, being the animals mostly used for this purpose. As we have seen, reports vary as to the pathogenicity of Influenza strains for this animal, some finding them lethal when injected in pure culture, others finding them lethal only when injected along with living or dead cocci (usually streptococci).

It is noteworthy that Hoyle (1932) when

in doubt as to whether a given Influenzoid organism was a "true" B. influenzae, used "the criterion of mouse pathogenicity as the deciding factor".

He found that nearly all strains which gave a weak oxidase reaction (on chocolate agar) with the tetra-methyl compound were almost invariably pathogenic to mice.

We carried out 4 different types of Experiments along these lines.

(I) The Leeds (control) Strains: tested (1) in pure culture and (2) along with dead Streptococci.

This gave a Standard of comparison.

(II) Tonsil Strains (24 selected at random), tested along with dead Streptococci.

(III) Tonsil Strains (6 selected at random), tested in pure culture.

(IV) Penicillin Broth Tonsil Cultures: (12 selected at random) after 24 hours incubation, inoculated in varying doses into mice. (a mixed infection) to compare results.

Technique used.

In all 65 mice were inoculated. We will here give a few details regarding technique, which are applicable throughout the whole series. This will save repitition.

I. Inoculation Route.

The intra-peritoneal route was used in

every case.

(2) Influenza Cultures. 24 hours cultures on Fildes agar slopes (6 "x½") (from Stocks) were always used; washed off in ½cc broth (+ Fildes blood digest 1%). This was warmed in incubator before injection.
Dose was either 1 Culture in 1/2cc (Fildes broth)
or ½ " in 1/4cc (" ")

(Only living cultures of course were used).

(3) Streptococcus used. This was a Stock strain of Streptococcus haemolyticus I - the same strain being used throughout. 24 hours culture on Hormone Agar (tubes 6"x½") was used, washed off in saline, ½cc for 1 whole agar culture. They were killed by standing in water bath at 56°C for 35 minutes.
Dose was either 1/2 agar culture in 0.25cc saline
or 1 " " in 0.5cc "

This was also warmed in incubator before injection.
Dead Streptococci were used in every case - This is understood where not specifically stated.

(4) Examination of Mice (P.M.) An autopsy was performed on all mice which died. Besides a general maked-eye examination; particular attention was paid to the peritoneal cavity for signs of Peritonitis; but cultures were not made from this. The condition of the spleen was noted (for evidence of Septicaemia). The most important examination however was the exposure of the heart (with aseptic precautions, and searing

exposed surface etc.) and removal of blood through a sterile capillary pipette, which was inoculated on to Fildes agar slopes. A direct smear of heart blood was also stained (in nearly every case) and examined for organisms (B. influenzae). The Fildes slopes were examined for growth after 24 hours - both naked eye and microscopically (films stained with Gram) in every case and results noted.

EXPERIMENT I

In this Experiment we tested the Control Cultures (sent by Prof. McLeod, Leeds) - 4 in all.

This Experiment is divisable into 2 parts which we will call Ia and Ib.

In the former the dose given of each of these Influenza strains was 1 whole culture (24 hours). In the latter the dose of these was 1/2 culture (24 hours). In all other details the experiments are identical.

(Note - The first 2 strains are typical, the other 2 atypical)

Experiment Ia.

Details are given in Table VI.

We note that one mouse (No. 2) died within 18 hours, and 2 died on the 3rd day, the fourth survived (possibly because culture was scanty).

Only the first yielded a positive Heart Blood culture (B. influenzae). These were inoculated with pure cultures, and we note the lethal strain was one which was known to be a True Pfeiffer. (McLeod).

The next 4 mice (5 to 8), however, all died, having been inoculated with the combination of Influenza bacilli + dead Streptococci. All died within 18 hours. The 2 controls survived (dead cocci only).

B. influenzae was grown from the heart blood of the mice inoculated with the 2 True Pfeiffer strains, but not from those inoculated with the 2 atypical strains.

From this Experiment we see that:-

(1) B. influenzae (Pfeiffer) can be lethal in pure culture (1 slant dose). and that

(2) its virulence is increased by injecting dead Streptococci along with it - This confirms the results of numerous observers.

Experiment Ib,

Details are given in Table VII.

This is identical with the above Experiment except that the dose of B. influenzae has here been halved - 1/2 culture (24 hours).

We find a marked contrast in the results. Here the mice all survived, except one, whose death appears to be due to accidental infection. The

organism grown from the heart blood was not like B. influenzae, although a Gram negative bacillus.

We conclude from this that ½ culture of these Influenza strains is a sublethal dose, whether alone, or combined with Streptococci. One whole culture appears to be the average lethal dose both in pure culture (effect more delayed), or along with dead Streptococci (effect more rapid).

EXPERIMENT II.

Details are given in Table VIII.

In this experiment we tested 24 of our Tonsil strains of Influenza bacilli, selected at random.

In every case one whole culture (24 hours) was the dose given, and dead streptococci (½slant culture) were also simultaneously administered. Controls with cocci only were included.

They were done in 3 batches of 6, 9, & 9, (1 control to each). Of the 24 strains tested, 22 proved lethal to the mice within 24 hours, and in every case the organism was retrieved from the heart blood, in pure culture, being identified by cultural and morphological characters as B. influenzae(group).

Direct Smears of heart blood were also stained and examined, and in these Influenza-like bacilli were frequently seen, sometimes few, sometimes

TABLE VI.

Mouse No.	Influenza strain used	Dose of Inoculum Influenza strain	Dead cocci	Result to Animal	Heart Blood Direct Smear	Heart Blood Culture.
1	"Cooper"	1 culture	nil	D3rd day	negative	negative
2	"Coates"	" "	"	D. 12-18 hours	"	Positive
3	"Noble"	" "	"	survived		
4	"Wheatley"	" "	"	D3rd day	"	negative
5	"Cooper"	" "	½cult	D. 12-18 hours	"	Positive
6	"Coates"	" "	"	D. " "	"	"
7	"Noble"	" "	"	D. " "	"	negative
8	"Wheatley"	" "	"	D. " "	"	"
9	Control	Streptococci only	"	survived		
10	"	" "	1 cult	"		

(D. = Died).

Regarding Mouse 3 "Noble" strain gave rather scanty growth on Fildes agar slope - reducing dose of inoculum. This may partly account for survival of this animal.

TABLE VII.

Mouse No.	Influenza strain used	Dose of Influenza	Inoculum Dead cocci	Result to Animal	Heart Blood Direct Smear	Heart Blood Culture
11	"Cooper"	½culture	nil	survived		
12	"Coates"	" "	"	"		
13	"Noble"	" "	"	"		
14	"Wheatley"	" "	"	"		
15	"Cooper"	" "	½cult.	"		
16	"Coates"	" "	"	"		
17	"Noble"	" "	"	"		
18	"Wheatley"	" "	"	D 12-18 hours	negative	Gram-bacilli not Influenzoid
19	Control	cocci only	½cult	survived		
20	"	" "	1 "	"		

abundant.

Well marked bipolar staining was noticed in several instances.

Gram's stain was used at first, and later Borax Methylene Blue. The latter was recommended by Prof. Cappell (Dundee), as being useful for showing capsules if present (A differential stain, capsules stain pink, organisms blue). We did not, however, find any with capsules. Both stains showed polar staining effect.

(Before staining with Borax Methylene Blue films were fixed (2 minutes) in a Reagent consisting of Formalin 1 part, Chloroform 3 parts, and Absolute Alcohol 6 parts).

One Strain, however, (No. 18) which was non-haemoglobinophil had been inadvertently included in this Experiment so that we have 21 out of 23 lethal Influenzoid strains. See Table VIII.

Of the 2 exceptions; Mouse 25 (Strain 49) died after 8 days; an organism was cultured from the heart blood, but was not B. influenzae. Mouse 39 (Strain 95) survived. Both of these may be regarded as negatives from the point of view of the Experiment. The survival of these mice may be attributed either to (1) lack of virulence on the part of the strains used, or (2) superior individual resistance (natural immunity) on the part of the mice.

We may conclude from this Experiment that 21 of our Tonsil strains (out of 23) have behaved like the "Cooper" & "Coates" strains (Mice 5&6) of Experiment Ia.-the conditions of the experiments being the same, i.e. they have behaved like typical strains of Pfeiffer' bacillus.

TABLE VIII.

Tonsil Influenzoid Strains + Dead cocci

Mouse No.	Influenzoid Strain used	Dose of Inoculum Influenzoid	Dead cocci	Result to Animal	Heart Blood Direct Smear	Heart Blood Culture.
21	No. 27	1 culture	½cult	D. 24 hours	not done	Positive Culture Influenzoid bacilli
22	" 36	" "	"	" "	" "	" "
23	" 45	" "	"	D. 12-18 hours	negative	" "
24	" 48	" "	"	" "	Positive Bipolar staining	" "
25	" 49	" "	"	D. after 8 days	negative	non-Influenzoid organism grown
26	" 54	" "	"	D. 12-18 hours	"	Positive Influenzoid bacilli
27	Control	cocci only Influenzoid	"	survived		
28	No. 64	1 culture	"	D. 12-24 hours	Positive Polar staining	Positive culture Influenzoid bacilli
29	" 69	" "	"	" "	" " "	" "
30	" 72	" "	"	" "	not done	" "
31	" 74	" "	"	" "	Positive	" "
32	" 78	" "	"	" "	negative	" "
33	" 81	" "	"	" "	Positive	" "
34	" 90	" "	"	" "	"Polar staining	" "
35	" 92	" "	"	" "	Positive	" "
36	" 94	" "	"	" "	"	" "
37	" Control	cocci only	"	survived		

Up to this point Gram's Stain was used.

TABLE VIII (Continued).

Mouse No.	Influenzoid strain used	Dose of Inoculum Influenzoid	Dead cocci	Result to Animal	Heart Blood Direct Smear	Heart Blood Culture.
38	No. 88	1 culture	½cult	D 12-24 hours	Positive Polar staining	Positive Influenzoid bacilli
39	" 95	" "	"	survived		
40	" 96	" "	"	D 12-24 hours	negative	" "
41	" 100	" "	"	" "	"	" "
42	" 15	" "	"	" "	"	" "
43	" 16	" "	"	" "	"	" "
44	" 17	" "	"	" "	"	" "
45	" 18	" "	"	" "	"	" "
46	" 19	" "	"	" "	"	" "
47	Control	cocci only	"	survived		

These direct smears were stained with Borax Methylene Blue.

No. 18 strain - a non-haemoglobinophil bacillus - was inadvertently included in this group

TABLE IX.

Tonsil Influenzoid Strains - Pure Cultures.

Mouse No.	Influenzoid strain used	Dose of Inoculum Influenzoid	Result to Animal	Heart Blood Direct Smear	Heart Blood Culture
48	No. 64	1 culture	D 36-48 hours	negative	Positive Influenzoid bacilli
49	" 81	" "	survived		
50	" 90	" "	D 12-24 hours	Positive Polar staining	" "
51	" 92	" "	" "	negative	" "
52	" 15	" "	survived		
53	" 19	" "	D 12-24 hours	"	" "

stained with Borax Methylene Blue.

EXPERIMENT III.

It was now decided to test the effect of injecting some of our Tonsil strains into mice in pure culture. We would have preferred to have carried this out with all the 24 strains used in Experiment II (for comparison of results) but owing to the fact that there were not enough mice available (and partly also because several of our Tonsil strains had died out or been discarded), we were obliged to restrict ourselves to testing 6 strains only.

The Dose in each case was one whole culture (24 hours). Results are given in Table IX.

We see that 4 of the 6 mice inoculated, died; 3 of them within 24 hours, the other within 48 hours. From all 4 the organsim (Influenzoid) was retrieved from the heart blood in pure culture.

The other 2 mice survived (strains 81 & 15).

We may conclude that those 4 lethal strains (Nos. 64, 90, 92, & 19) have behaved like "Coates" Strain in Experiment Ia (Mouse No. 2) i.e. like a True Pfeiffer Strain when injected in pure culture, the conditions of the experiments being the same.

CONCLUSIONS.

Taking these results of Experiments II & III together, (the strains having all been chosen at

random) they suggest that a not insignificant proportion of our Tonsil Strains behave exactly like true Pfeiffer bacilli in regard to mouse pathogenicity.

We have seen that Hoyle regards this as the deciding factor when in doubt about any given strain being true Pfeiffer. We shall see however, that our Mouse pathogenic strains did not all give a weak positive oxidase test, and that in their growth requirements, and Tellurite Tolerance, they also showed great variation.

Conclusions from Experiments I, II, & III.

(1) B. influenzae (Pfeiffer) can be lethal to the mouse in pure culture and the organsim can be recovered from the heart blood.

(2) Its virulence is enhanced by simultaneous injection of dead streptococci.

(3) Different strains of Pfeiffer bacillus (typical) may vary in their virulence to the mouse (in pure culture) (cf. mice 1&2).

(4) The minimum lethal dose of these typical strains was found to be approximately 1 tube culture (24 hours).

(5) Atypical strains appear to be less virulent than the typical ones, (When lethal the organism does not seem to be so readily recovered from the

heart blood).

(6) Of 23 tonsil Influenzoid strains tested 21 were lethal to the mouse when dead cocci were simultaneously injected. The organism was recovered from heart blood in every case.

(7) Of 6 tonsil strains tested 4 were lethal to mice in pure culture. (Dose = 1 tube culture, 24 hours). In all 4 the organism was recovered from the heart blood.

(8) As regards mouse pathogenicity we conclude that a not insignificant proportion of our tonsil haemoglobinophilic strains behave like typical Pfeiffer's bacillus.

EXPERIMENT IV.

In this experiment we used our cultures of Penicillin broth + Tonsil tissue as the inoculum. These provided mixed cultures which we knew contained Influenza bacilli and also Gram negative cocci (Flavoid). We presumed that some Gram positive cocci might also have survived. We used the cultures after 24 hours incubation. We then examined them micrsocopically and found that Streptococci were present in every instance (with 1 exception).

We inoculated 12 mice with these, using 12 different Tonsil cultures. The dose of inoculum was also varied. Four received 0.25cc, 6 received

0.5cc and 2 received 0.75cc. - all given intraperitoneally.

Results here are not included in Tabular Form as they are of secondary importance.

Four of the 12 mice died within 36 hours - 2 inoculated with 0.5cc and the 2 with 0.75cc. Organisms were cultured from the heart blood in every case (mixed infection). In one, the predominating organism was Friedlander's bacillus, in the other 3 Influenzoid (tiny Gram negative bacilli) and Flavoid (Gram negative cocci) were conspicuous, the former predominating in 2 cases, the latter in one case. A large Gram negative bacillus was also present in 2 of these, and Streptococci in only one case. No other Gram positive cocci were seen.

The other 8 mice survived, except 2, which died from causes which appeared to be accidental.

One of these lethal cultures was from Tonsil 95, and we have seen that Influenzoid strain 95 was non-lethal in association with dead streptococci. Here it is lethal in association with living Flavoid cocci. We cannot be sure however, which of the organisms recovered, plays the greater part in producing the lethal effect.

CONCLUSIONS. EXPERIMENT IV.

(1) Penicillin broth + Tonsil tissue cultures

(24 hours) used as inoculum may prove lethal to mice.

(2) The lethal dose is from 0.5cc to 0.75cc, given intraperitoneally.

(3) Various organisms are recovered from the heart blood - showing a mixed infection (Septicaemia).

(4) Influenzoid bacilli and Flavoid cocci were the predominating organisms recovered; but large Gram negative bacilli were also frequently present.

(5) It is impossible to decide which organisms played the greatest part in producing the lethal effect.

(6) That the virulence of the Influenzoid bacilli is enhanced by the presence of Gram negative (Flavoid) cocci is suggested in at least one case.

Having completed the tests set out on page 132 we submit in conclusion a Table X showing the 24 Tonsil Strains used in the Mouse Pathogenicity test, giving results of this test, the growth requirements of these strains, and also their reaction to the oxidase and Tellurite Tolerance Tests.

It will be seen that many strains giving a negative oxidase reaction are pathogenic to mice (at least along with dead cocci). This differs from Hoyles finding (referred to on p.149) which correlates a positive oxidase reaction and mouse pathogenicity. It so happens that the 4 strains (Nos. 64, 90, 92, & 19) which were lethal in pure culture all gave a very strong oxidase reaction and 3 of them required only X factor.

This suggests the possibility that strains which demand X factor only, may be more pathogenic to mice - but with our small numbers it may be only coincidence. We also note (From Table X) that strains which demand both X & V may vary considerably in their pathogenicity to this animal e.g. Strain 19 is lethal both in pure culture, and along with dead cocci, Strain 15 is not lethal in pure culture but is lethal along with cocci, whilst strains 49, & 95 are not lethal even along with dead streptococci.

We regret we were not able to inoculate a larger series of mice with pure cultures of our tonsil strains.

On carefully examining results in Table X. we are unable to find any correlation between growth requirements, oxidase reaction, Tellurite tolerance, and Mouse Pathogenicity. These tonsil strains appear to be a heterogenous group of organisms - so far as these combined tests are concerned.

TABLE X.

Strain No.	Growth Requirements	Oxidase reaction	Tellurite Tolerance Test	Mouse Pathogenicity (a) along with dead cocci	(b) in pure culture.
27	X + V	—	C	lethal	
36	" " "	—	A	"	
45	" " "	—	—	"	
48	" " "	++	C	"	
49	" " "	++	A	non-lethal	
54	" " "	—	A	lethal	
64	X only	++	C	"	lethal
69	X + V	—	C	"	
72	XorV (?mixed)	++	A	"	
74	X + V	+	A	"	
78	V only	—	A	"	
81	" "	+	A	"	non-lethal
88	X + V	++	A	"	
90	X only	++	A	"	lethal
92	" "	++	A	"	"
94	X + V	++	A	"	
95	" " "	—	C	non-lethal	
96	V only	+	C	lethal	
100	" "	weak+	A	"	
15	X + V	—	A	"	non-lethal
16	" " "	weak+	B	"	
17	" " "	+	B	"	
18	Non-haemoglob-inophil	weak+	B	"	
19	X + V	++	B	"	lethal

In above list only 1 strain fulfils Hoyle's conditions for being a true Pfeiffer bacillus-namely No. 16-i.e. weak Oxidase positive & Mouse pathogenic. Two other strains almost fulfil it-Nos. 74 & 17 but oxidase test more strongly positive. No. 100 fulfils these 2 conditions-but demands Vfactor only. Strains 81 &96 behaved similarly.

Summary & Conclusions Section II.

(1) From 100 tonsils 80 strains of haemoglobinophilic bacilli (Influenza group bacilli) have been isolated by the Penicillin technique described.

(2) The effectiveness of Penicillin for inhibiting Gram positive organisms generally (as recommended by Fleming) has been corroborated. The Influenza group bacilli appear to be insensitive to its presence.

(3) In classifying these tonsil strains by their growth requirements we find 55 strains demand X & V factors, 14 strains demand V factor only, and 7 strains demand X only - (the other 4 being ?mixed or doubtful).

(4) Colonially and morphologically all the 80 strains appear to be typical. None were haemolytic. All of them suspended easily in saline. No filamentous forms were seen.

(5) With regard to Oxidase reaction; this was very variable. e.g. of 41 strains demanding X & V factors, 20, gave a positive reaction (varying in degree) and 21 a negative. No correlation between this test and the nutrient requirements of the strains could be established. This contrasts with the findings of McLeod and Hoyle already referred to.

(6) Similarly the Tellurite Tolerance of the strains varied considerably, and did not seem to bear any definite relationship to their growth requirements.

Our chief finding was that the majority of the strains showed a marked degree of tolerance to this reagent. e.g. 37 of the 80 strains withstood Tellurite in 1/20,000 concentration (Controls behaving likewise) and 77 of the 80 strains withstood it in 1/80,000 or stronger.

(7) Our Mouse Pathogenicity tests suggested that a significant proportion of our Tonsil strains behaved (in this respect) like B. influenzae (Pfeiffer); being lethal to the mouse in pure culture; and their virulence being enhanced when dead streptococci were injected simultaneously.

(8) We have seen that an outbreak of mild Influenza occurred whilst this work was proceeding - but that no fluctuation in the incidence of these Influenza bacilli in Tonsils was observed. They appeared to be practically universally present before, during, and after the outbreak. A large number of children were apparently attacked by this Influenza - as evidenced by school attendance during January, 1933. This fell to an exceptionally low level in the 3rd week of that month, but returned to normal in February.

(9) The question arises - Are these Tonsil Influenzoids pathogenic or non-pathogenic to man? Were they connected etiologically with the mild Influenza epidemic mentioned? - or are they harmless commensals ("haemophilic weeds ") forming part of the basic flora

of the mouth and throat, but resembling B. influenzae (Pfeiffer) in their growth requirements, etc,?. If the latter assumption be correct then their pathogenicity for mice, the fact that they suspend easily (in saline), and their various other reactions render them (most strains at least) indistinguishable from the true B. influenzae. Fleming and McLean (1930) state that "the name B.influenzae is applied to a number of different bacteria which have grossly different cultural and morphological characters, and which appear to vary widely in their pathogenicity. No one will deny a pathogenic role to the true Pfeiffer bacillus but it has yet to be established that these normally occurring variants are concerned with any infective process".

Amongst our strains we did not observe any which showed "grossly different cultural and morphological characters" from B. influenzae. We were impressed rather with the very close resemblance of many of our strains all through to the Pfeiffer bacillus. There is no information as to whether these strains isolated by Fleming and McLean were pathogenic to animals.

(10) To sum up, we seem to be dealing with a group of organisms many of which are either (a) identical with the true B.influenzae (according to the tests performed) or (b) closely related strains, indistinguishable from the Pfeiffer bacillus by any tests

within our present knowledge.

If the latter conclusion be accepted, we may regard these tonsil strains as differing from true B. influenzae in some inherent quality by which the latter is either actually or potentially pathogenic to man, whilst the former are presumably non-pathogenic and appear to be universally present in normal throats. Regarding their non-pathogenicity we have no proof of this. They may be like the ubiquitous streptococci and pneumococci --- potential pathogens.

McLeod states (private communication) that Hoyle finds that at least 90% of the Pfeiffer strains, which are capable of killing the mouse in pure culture, come from inflamed mucous membranes. Whether "enlarged and diseased tonsils" would be included under this description we are not certain, but if so, our mouse experiments would seem to agree with this observation. "Sore throats" were a frequent occurrence in the children concerned, and such tonsils are frequently the seat of a subacute or chronic inflammatory process.

It is apparent that in the present state of our knowledge we can come to no final conclusion regarding these Influenzoid organisms isolated from tonsils. It would be of interest to know e.g. the incidence of these organisms at different seasons of the year, and during periods free from Influenza; also whether they are found as frequently in children who have not "enlarged tonsils". Much more research along various lines will be required to elucidate this complex subject.

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SECTION 111.

To determine the incidence of Haemolytic Streptococci in Extirpated Tonsils.

Section III.

The Occurrence of Haemolytic Streptococci in Tonsils.

INTRODUCTION.

As the material was available we decided, whilst examining tonsils for other organisms, that it would be of interest to include also a superficial study of the streptococci present in these organs - limiting ourselves to the Haemolytic Streptococci.

These Haemolytic strains form the most important group of the Streptococci since they are generally speaking, more pathogenic than the other groups, and have been proved to be responsible for a variety of human infections.

A preliminary experiment was done to decide what technique would best answer our purpose.

The criterion of haemolysis was the definitive test of McLeod, using serum broth cultures (18 hours) in a series of dilutions.

A few references to the literature will first be given, dealing with such as are relevant to our present superficial survey.

Part I. Review of the Literature.

Streptococci are found in different parts of the body, as well as external to it, and thus we

speak of skin streptococci, mouth streptococci, and bowel streptococci, etc., according to their natural habitat.

A satisfactory classification of the Streptococci is however, one of the problems of bacteriology which still awaits solution.

It is generally agreed that Serological methods have proved unreliable for this purpose, as it was not found possible to separate them into definite types, as for example, in the case of the Pneumococcus.

Differentiation by fermentation reactions (sugars) has been elaborated (Holman's classification) and is used to some extent, but has not proved completely satisfactory.

The test which has proved most useful so far in subdividing this genus of organisms is that of haemolysis. For practical purposes they are divisible into haemolytic and non-haemolytic groups. This subject has been most extensively investigated by Brown (1919).

He divides Streptococci into 3 groups; according to the appearance of the colonies, when grown on solid blood agar medium (plates). It was Schottmüller's (1903) introduction of the blood agar plate which led to the differentiation which we are discussing, and to a wider use of the haemolytic

criterion (McLeod 1929).

The 3 groups are as follows:-

α type: The common green-producing Streptococci with slight clearing about the colony, but no real haemolysis.

β type: Colonies are surrounded by a broad well defined zone of clearing 2-4mm diameter.

γ type: The indifferent or grey Streptococci which produce neither green discolouration nor haemolysis.

Still another type, the α prime type: must be noted which, according to Bloomfield & Felty (1923) can only be distinguished from the β type by using the low power of the microscope. Intact R.B.Cs. are seen and some haziness may also be present. But whilst the β type is pathogenic, the α prime type is non-pathogenic,- yet both look very similar on blood plates. (Bloomfield & Felty 1923).

The α type correspond to what is generally called Streptococcus viridans, so commonly found in the mouth.

We see therefore that the blood plate test for haemolysis may not be quite accurate, and so a finer test has been introduced - (McLeod's test - quoted by OKell 1932). Haemolytic Streptococci are referred to as being those which will, when grown in serum broth for 15-18 hours produce a haemolysin which can be detected by incubating a small quantity of the

culture fluid with a suspension of Red blood corpuscles in 0.9% saline for about 1½ hours (at 37°C). A shorter incubation may suffice e.g. ¾ of an hour (McLeod 1921). A series of dilutions of the Streptococcus culture is used (as in ordinary agglutination tests) and thus a "Haemolytic titre" may be obtained.

This Definitive Test has not been used by all observers. Most reports regarding incidence of haemolytic streptococci seem to be based upon the less accurate blood plate method. According to OKell (1932) however, the discrepancy between the 2 methods is not great. He states that the Streptococci causing β haemolysis on plates give in the great majority of cases a positive McLeod test, but whereas the interpretation of the peculiar appearances met with on plating often proves difficult, the result given by the McLeod test is nearly always clear. He adds, "In a recent series of comparisons carried out on 76 strains of "haemolytic" streptococci, using rabbit blood for both tests, I only met with 2 strains in which a true contradiction occurred between the 2 tests, one McLeod test positive and plate test negative, and one the reverse. Four strains were doubtfully haemolytic with both tests."

<u>The Pathogenicity of Haemolytic Streptococci to Man.</u>

As we have already noted there appears to

be some connection, speaking generally, between Pathogenic power and haemolytic action in these organisms. The haemolytic streptococci are associated with a variety of infective processes in man. The non-haemolytic strains (usually S.viridans) can also be pathogenic but to a much smaller extent and they tend to produce chronic conditions rather than acute ones. The pathogenic effect of all types depends upon the virulence of the individual strain, and the susceptibility of the host.

Amongst the commoner conditions associated with haemolytic streptococci are the following:-

Wound suppuration; localised abscesses; cellulitis; erysipelas; septicaemia (e.g. puerperal septicaemia); acute osteomyelitis; otitis media and mastoiditis (suppurative); tonsillitis and pharyngitis e.g. (acute follicular tonsillitis); the angina of Scarlet Fever; and broncho-pneumonia complicating Influenza, Measles etc., (Mackie & McCartney 1928).

It appears to be generally accepted (since the work of the Dicks 1921, 1924) that Scarlet Fever is due to infection with a haemolytic streptococcus which invades the fauces, producing a diffusable toxin, responsible for the rash and other symptoms. The Streptococcus may invade the blood stream and viscera (septicaemic form) in fatal cases.

In Measles cases Cole & McCallum (1918) found that relatively few had haemolytic streptococci in their throats on admission to hospital, but after a short stay in hospital the number of positive throats was greatly increased. They found that most of the complications (Broncho pneumonia & otitis media) were due to haemolytic streptococci, and they concluded that these were largely due to the hospital environment. - They compare them to the puerperal and wound infections which are often traceable to the same environment - and sometimes due to medical or nursing attendants being carriers of haemolytic streptococci in their throats.

Beattie (1921) points out that haemolytic streptococci are found in the throats of patients suffering from several conditions e.g.

Scarlet Fever.	131 cases	91% positive
Measles	538 "	72% "
Influenza	340 "	70% "
Lobar Pneumonia.	151 "	50% "

These figures represent the average of several observers. These streptococci are regarded as the chief cause of the complications in these and other conditions.

He also mentions that the percentage of positive throats in normal persons is greater where they are aggregated together, quoting Levy & Alexander

who found that in recruits joining camp, 14.8% had these streptococci in their throats, whilst in healthy men after 6 months in camp the figure rose to 83%.

These various observations raise the question of "carriers" of these haemolytic streptococci which will now be examined.

The Occurrence of Haemolytic Streptococci in Normal throats and in extirpated Tonsils

Davis (1919) & (1920) states that S. haemolyticus is almost constantly found in the crypts of the tonsils, but not quite so frequently on the surface of these organs. In 55 of 61 Tonsils from children, he found it in the crypts i.e. in 90%.

He quotes Pilot who examined 100 tonsils comparing the incidence of this organism in 3 localities. His findings were:-

in crypts 97% gave haemolytic Streptococci
on surface of tonsils 61% " " "
in pharymx (before extirpation) 43% gave haemolytic Streptococci.

In another series of 24 normal throats 58% were positive. In 19 persons without tonsils only 15% were positive.

Davis concludes that the tonsil crypts are

an almost constant source of haemolytic streptococci, and that this may be their normal habitat. He knows no other structure in the body which so constantly harbours them.

N. Wall (1922) examined over 100 Tonsils and Adenoids from children and divided the Streptococci into 3 groups as follows:-

(a) those showing complete haemolysis (β type) = 84%
(b) " " partial " (?α type)= 34%
(c) " " no " (γ type) = 92%

She noticed also that the first group were more frequently found in the crypts than on the tonsil surface.

Felty & Hodges (1923), in a study of acute follicular tonsillitis, isolated β haemolytic streptococci from practically 100% of cases. These are generally accepted as the causal organism of this condition. These authors conclude that the "chronic carrier" of the haemolytic streptococcus, shown by many observers to constitute a large percentage of the community is the abvious focus of infection from which new cases develope, as in the spread of Typhoid fever etc.

They add "It is not due to auto-infection from the patients' own tonsils. If he carries the S. haemolyticus in his tonsils this rather gives him

immunity to such acute infection".

Nakamura (1924) in an examination of over 2,000 extirpated Tonsils (during 12 consecutive months) found Streptococcus haemolyticus the predominating organism in the crypts. He gives his findings for the 3 sub-groups as follows:-

(1) Haemolytic Streptococci (β type) = 52%
(2) Green producing " (α type) = 58%
(3) Indifferent " (γ type) = 18%

He believes there is an increased incidence of haemolytic Streptococci in normal throats during the winter months; (the season when tonsillitis and angina are most common).

Eves & Watson (1925) made a study of the incidence of S. haemolyticus in Tonsil crypts, taking swabs from normal throats (no tonsillitis or hypertrophy), They examined different classes of people - Students, private patients, dispensary patients, - and found the positives varied from 21% to 84%; the average of 6 groups being about 61%.

These authors also conclude that the natural home of the S. haemolyticus is the Tonsil crypts.

They discuss the significance of these organisms in the tonsils, and the possible role they may play in either the prevention or cause of disease.

They consider that there is good evidence that β-haemolytic streptococci play a very beneficient part in the prevention of acute tonsillitis. They agree with Felty & Bloomfield who found that tonsil carriers of these organisms failed almost uniformly to develope tonsillitis, whereas the incidence of the disease in those who were not carriers was over 40%. In brief "Carriers of β-haemolytic streptococci are practically immune (to acute tonsillitis); whilst non-carriers are highly susceptible."

In a special investigation in Manchester, conducted from July, 1925 to September, 1927, Topley (1928-30) reports that "the nasopharyngeal carrier - rate for haemolytic streptococci rose fairly steeply from a low level (3.2%) in July, 1925, to a peak (36.4%) in November of that year. It then fell slowly and steadily to a level of 3.7% in May, 1926, fluctuated between 8.5% and zero until October, 1926, and then showed a slight and gradual rise, with irregular fluctuations until September, 1927, the last month of the enquiry, when it reached a value of 25%".

They add that it was obviously possible that the frequency of these haemolytic streptococci was determined largely by the prevalence of Scarlet Fever (in Manchester), in spite of the fact that the persons examined were of an age when the susceptibility

to Scarlatinal infection is relatively low.

It is noteworthy that these statistics of Topley are considerably lower than those previously quoted for other observers.

Polvogt & Crowe (1929) investigating 100 Tonsils and Adenoids (extirpated) found S.haemolyticus the predominant organism in 91%. They thought this organism was relatively more common in children than in adults.

Cobe (1930) in an examination of 400 Tonsils found that on an average 20.75% were positive for haemolytic streptococci, whilst Staptylococci predominated in 68.5%. He confirmed Polvogt & Crowe's observation that there is a relationship between the type of organism recovered from tonsil cultures and the age of the patient - the Streptococci being more frequent in younger patients (under 11 years). He also observed a seasonal difference in the organisms recovered.

Nabarro & MacDonald (1929) examined 2 series of tonsils from (a) rheumatic and (b) non-rheumatic cases; comparing the Streptococcal flora in each. They found them very similar in practically all respects, including the haemolytic strains which were present in 14% of both series. We may note here that the theory of the streptococcal origin of

Rheumatic Fever (made prominent by Poynton & Paine in 1900), is still a matter of uncertainty. Most strains isolated from cases appear to belong to the non-haemolytic group.

Glover & Griffith (1931) reported relatively high carrier rates during Scarlet Fever outbreaks (in Schools) e.g. 33, 26, 26, 36, & 33 - % in 5 instances; but they note that as high a rate was met with when there was no Fever about, e.g. 27% during vacation. "Scarlet Fever is an infection in which the ratio of carriers to typical cases is high".

Besides human carriers we may in conclusion mention one other source of infection with haemolytic streptococci - namely, cow's milk. Outbreaks of sore throat have sometimes been traced to milk from cows suffering from streptococcal mastitis; and there appears to be evidence that Scarlet Fever has occasionally been a milk-borne infection. But human sources of infection (carriers of haemolytic streptococci) have to be considered as possible in such cases, i.e. contamination of the milk.

Jones & Little quoted by McLeod (1929), however, have traced a milk-borne outbreak of Scarlet Fever to cows whose udders were naturally infected with scarlatina streptococci.

Bovine types of haemolytic streptococci are also found in milk and appear to be mostly non-pathogenic to man (Frost, Gumm, & Thomas (1927) and others).

The Present Investigation

Introduction.

As in the case of Br. abortus and B. influenzae we found it necessary here also to make preliminary investigations in order to find out what technique would serve best for the isolation of haemolytic streptococci from Tonsils.

During our preliminary experiments in Section I (Br. abortus) we noticed that the various Gram positive cocci of the tonsils were inhibited by different concentrations of Crystal Violet - i.e. in the presence of tonsil tissue.

Our findings were approximately as follows:-

C.V. 1/10,000 inhibits Staphylococci, Streptococci, & Pneumococci.

C.V. 1/20,000 - 1/40,000 inhibits the first 2, but allows Pneumococci to develope

C.V. 1/40,000 - 1/80,000 inhibits Staphylococci but allows Pneumococci and Streptococci to develope.

We knew that Potassium Tellurite had an inhibitory action upon the growth of Influenzoid

organisms (although we had not yet completed our Tellurite Tolerance Tests).

We also knew that Optichin (Ethyl hydrocupreine) is lethal to pneumococci in low concentrations 1/300,000 - 1/1,000,000. Streptococci are much less susceptible. (Prof. Tulloch, personal communication)

We decided therefore to carry out a preliminary experiment using these 3 reagents in suitable concentrations, C.V. to inhibit Staphylococci, Tellurite to inhibit Influenzoids, and Optichin to restrain Pneumococci - in the hope that isolation of Streptococci might be facilitated by this procedure.

Preliminary Experiment I.

Using Tonsils 31-36 (gauze filtrate) we inoculated 0.2cc of each into 2 series of broth tubes as follows (12 in all):-

Series A Broth + Crystal Violet 1/40,000
+ Tellurite 1/40,000
+ Optichin (base) 1/250,000

Series B Broth + Crystal Violet 1/40,000
+ Tellurite 1/40,000
(No optichin).

These were incubated for 3 days at 37°C then subcultured to Rabbit Blood agar plates; and

the tube cultures were also examined microscopically and showed mostly pleomorphic Gram negative bacilli (?Influenzoids) and Gram positive cocci (?Streptococci). The Blood agar plates were examined after 24 hours naked-eye and microscopically.

RESULTS.

In both series (A &B) tiny colonies (numerous) were equally present - ? Pneumococci or Streptococci.

Pleomorphic Gram negative bacilli were met with in both series, showing curious swellings. We were not certain of their identity but considered they might be Influenzoids showing atypical morphology. We had not met such forms with our Penicillin technique. Staphylococci (haemolytic) came through in one instance.

By this technique however, we were only able to isolate haemolytic streptococci in one case (No. 31).

From this experiment we deduced the following:- (1) that the presence of tonsil tissue in the culture is weakening the effect of the Tellurite and optichin just as it does with Crystal Violet.

(2) that the Tellurite 1/40,000 is too weak, as Gram negative bacilli

(?Influenzoids) are coming through.

(3) that Streptococci come through quite well with optichin present. (apparently about equally well in both series).

EXPERIMENT II.

We decided to try a simpler technique and compare results with the above more elaborate one. We accordingly with the next lot of tonsils (37-42) inoculated a very tiny loopful (of gauze filtrate) directly on to Blood agar plates. With a tiny amount of inoculum and spreading it well, it was possible that resulting colonies might be sufficiently spaced out to allow of isolation of suspected ones.

On examining the blood agar plates after 24 hours, we found Streptococcal-like colonies - some haemolytic - on all 6. We isolated and subcultured one haemolytic colony from each plate, on to Ordinary agar (tubes). After 24 hours incubation these were examined naked-eye and microscopically (Gram stain) to see if they were streptococci and pure culture.

RESULTS.

Five out of these 6 yielded a pure

streptococcal culture. In No. 41 the growth resembled Pneumococci so this one was repeated (from tonsil filtrate kept in ice-chest), the second attempt being successful..

We had therefore succeeded by this simple direct technique in isolating 6 strains of ?haemolytic streptococci from these 6 tonsils examined (37-42).

Technique adopted.

We concluded that the direct technique of Experiment II was not only simpler but gave better results than that of Experiment I. We therefore adopted the former as our routine method for this investigation. The details were as follows:-

(1) Inoculate Rabbit blood agar plates directly with Tonsil (gauze filtrate); using tiny amount of inoculum and spreading carefully.

(2) After 24 hours incubation (37°C) isolate and subculture haemolytic streptococcal-like colonies on to Ordinary agar slopes.

(3) After a further 24 hours incubation examine resulting growth to see if it is streptococcal in character and pure culture.

(4) Make Stock culture of all strains thus isolated.

Stock cultures were prepared by simply making a stab inoculation of each strain into semi-solid agar (in tubes). These were kept for further investigation and renewed every 10 days.

Growth on Rabbit Blood Agar Plates.

As would be expected these plates presented a variety of colonies representative of the varied flora found in the tonsils. Streptococcal-like colonies were present in the majority of the plates - but a large number of these showed the α type of colony (S. viridans). Green pigmentation was present in approximately 60% of the plates; we did not attempt to differentiate between Streptococci and pneumococci in these cases - as both produce the "viridans effect" (due to Methaemoglobin production).

Our chief difficulty arose from Staphylococci which in several instances overgrew everything else, and frequently made isolation of suspected streptococcal colonies difficult. This was sometimes due to over-inoculation; and second, or third attempts were sometimes made, inoculating more lightly.

Use of Crystal Violet.

When the direct method failed, especially

after a second attempt, and where failure was due to predominance of Staphylococci, we employed the Crystal Violet technique to see if this would facilitate isolation of haemolytic streptococci by inhibiting these other cocci. (Haxthausen 1927).

On the whole, however, this was not very successful. We used concentrations of 1/20,000 and 1/40,000 (in broth), inoculated with 0.2cc Tonsil (gauze filtrate) - but also tried a smaller dose of inoculum. After(usually) 2 days incubation subcultures were made to blood agar plates, which were examined after 24 hours.

In most instances the resulting growth consisted of S. viridans (?Pneumococci). In only 3 cases out of about 30 were we successful in isolating a true β-haemolytic streptococcus by this method.

The impression gained during this investigation was that S. viridans is apparently more resistant to Crystal Violet than are the true haemolytic strains. We did not, however, pursue this matter further.

Colonial Appearances & Morphology.

Where the tiny well defined Streptococcal like colonies, surrounded by a definite broad zone of clearing, were present, these were chosen for

isolation and subculture wherever feasible. In the absence of these, doubtful looking colonies were sometimes isolated, - and also some α type colonies to compare results with McLeod Test.

In this way we collected 67 strains altogether from 100 tonsils examined.

Regarding microscopic appearances our strains showed considerable variation. Chains varied in length from short to very long; and the individual cocci showed both round forms (the majority) and elongated ones (especially in long chains). The size of the individual elements also varied in different strains, some being very small, others comparatively large.

McLeod's (Definitive) Test.

All our strains were submitted to this test. Each strain of Streptococcus was grown in serum broth (0.4cc rabbit serum + 0.2cc broth) for 16-18 hours. A series of dilutions (in ordinary agglutination tubes) of each culture was made, and to each was added a 3% saline suspension of fresh rabbit blood corpuscles; to make the following series of final dilutions:-

1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128.

These tubes were then placed in the water bath at 37°C for 1½ hours, when the results were recorded.

When positive a pink colour is diffused throughout the tube contents (haemoglobin set free); whilst negative tubes show a dark deposit (R.B.Cs) at the bottom, with clear colourless fluid above (no haemolysis).

A result was regarded as positive if haemolysis was present up to dilution 1/8 or higher.

RESULTS.

A positive McLeod Test was present in 36 of our strains. Hence 21 of the 67 strains tested were negative.

In comparing the results of this Definitive Test with those of the blood agar plate test - we noted:-

(1) that 4 of our strains which gave a positive plate test, gave a negative McLeod test. (? α prime colonies).

and

(2) that 2 of our strains which gave a negative plate test (or at least showed only partial haemolysis) gave a positive McLeod Test. The titres of these strains were 1/64 and 1/128.

Haemolytic Titre: Regarding Haemolytic Titres of these 36 positive strains we found the following:-

20 strains were positive up to 1/128 dilution
11 " " " " " 1/64 "
2 " " " " " 1/32 "
1 " " " " " 1/16 "
2 " " " " " 1/8 "

Production of Methaemoglobin:- A greenish brown colour was noted in many of the tubes where haemolysis had occurred. No methaemoglobin was present in the negative tubes. (except only a trace in 2), 17 strains showed this methaemoglobin production quite definitely, and 5 gave it slightly. Whilst most marked in the lower dilutions it was present in some cases up to the weakest dilutions. In 14 positive strains no methaemoglobin was produced.

Summary & Conclusions Section III.

(1) Haemolytic Streptococci were obtained from 36 out of 100 tonsils, using the direct (blood agar plate) technique described for their isolation.

(2) The criterion of haemolysis was the definitive test of McLeod.

(3) Four strains gave a positive plate test and negative McLeod test; and 2 the reverse.

(4) Our finding is high compared with some e.g. Wall (1922), Nabarro & MacDonald (1929), & Topley (1928-30), but low compared with others e.g. Davis (1919), Pilot (quoted by Davis) Nakamura (1924), Eves & Watson (1925) and others.

(5) Topley's maximum finding during fully 2 years was 36% (adults mostly examined). It was suggested that the prevalence of Scarlet Fever might have largely determined the incidence of haemolytic Streptococci in these normal people (adults) (p. 184). This would presumably be true also in the case of children perhaps more so since they are more susceptible to scarlatinal infection.

Cases of this Fever were occurring in Dundee and district during the period of our present investigation, but not, so far as we are aware, to any unusual extent.

(6) We have the following data available regarding 22 of the 36 children whose tonsils yielded haemolytic streptococci in our series.

(a) only 3 (14%) have a history of having had Scarlet Fever.

(b) 10 gave a history of sore throats (usually recurring) (=45%), and in 3 others the tonsils were diagnosed as "Septic" before

operation. Follicular tonsillitis had been observed in 2 of the above 10, and repeated Tonsillitis in another.

(c) In 15 of the 22, enlargement of the cervical glands had been observed in varying degree i.e. in 68%. It was quite marked in 4 cases.

(7) Taking a similar group (20 cases) which were negative for haemolytic streptococci we find the following:-

(a) 2 cases had a history of Scarlet Fever (10%).

(b) 4 gave a history of sore throats (=20%) and 2 others had Septic Tonsils. Follicular Tonsillitis was recorded in 1 case and simple Tonsillitis in another (in addition to those with sore throats).

(c) In 7 of the 20, enlarged cervical glands were noticed i,e. 35%, but in none was it of marked degree.

(8) Comparing results in (6) & (7) above we note the fact that the Positive group (carriers of S. haemolyticus) give

(a) a history of sore throats.

and (b) evidence of cervical adenitis, approximately twice as frequently as do the negative group (non-carriers of S. haemolyticus).

(9) Regarding (a) history of sore throats

Since sore throats and tonsillitis are associated so frequently with S. haemolyticus infection we might expect such a finding. Our result therefore would agree with the etiological role of this Streptococcus in such throat conditions in children.

With regard to (b) cervical adenitis.

The tonsils are no doubt the portal of entry for organisms invading the cervical lymphatic glands and causing this condition. Our finding suggests that haemolytic streptococci may play a part in producing this common clinical condition.

(10) Our numbers are too small to allow any definite conclusions; but the relationship between bacteriological findings and clinical conditions may be regarded as suggestive.

It would be useful to know whether any seasonal fluctuation occurs in the incidence of these haemolytic streptococci in children, also the

frequency in children who are aggregated together (e.g. in schools). This might be expected to be considerably higher.

As in the case of our Influenzoid results, so here also, wider investigations are very desirable.

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SECTION IV.

The Occurrence of certain other Organisms Encountered, though not Specifically sought in Extirpated Tonsils.

Section IV.

(a) To determine the frequency in which B. tuberculosis can be recovered from tonsil tissue.

Introduction.

As we were inoculating guineapigs with tonsil tissue (gauze filtrate) to find out how frequently B. abortus could be recovered, it was considered desirable to look out at the same time for the presence of B. tuberculosis, as the same technique would suffice, and the same inoculated animals would record the presence of either organism by manifesting the lesions or reactions typical of each form of infection. We have seen what these are in the case of Abortus disease in guineapigs. As evidence of tuberculous infection we looked for signs of general emaciation, enlarged or caseous lymphatic glands, tubercles or caseous nodules (or abscesses) in the spleen, liver, and lungs, etc. Smears were made, especially from affected lymph nodes, and stained with Zeehl Nielsen, to look for the acid fast bacillus (B.tuberculosis).

For this purpose the guineapigs were kept 6 to 8 weeks before being killed, to allow more opportunity for tuberculous lesions developing, should tubercle bacilli be present in any of the tonsils in minimal amount.

Morton (1933) showed that guineapigs may be negative (for Tuberculosis) at 4 weeks but positive

after 8 weeks.

Wilson and Nutt (1926) have shown that both organisms (B.tuberculosis and B.abortus) might infect the same animal simultaneously, and that both might be recovered from it. They accomplished this in 9 cases (milk being the inoculum). They tested for B. tuberculosis by microscopic examination and for B. abortus by Culture.

Secondary tuberculosis of the tonsils occurs frequently ~~and~~ in advanced pulmonary phthisis from the sputum) in adults (being uncommon in children who swallow their sputum). We are concerned here, however, only with primary tuberculosis, and latent tuberculosis of the tonsil in children who have no signs of the pulmonary infection.

Part 1. Review of the Literature.

We have selected a few of the more important reports of investigations bearing on our subject. These we believe will suffice as a basis for comparison with our own results.

Amongst the earliest observers to report the condition which we may call "Latent Tuberculous infection of the Tonsils" were Lermoyez in 1894 (quoted by Vlasto) and Dieulafoy in 1895 (quoted by Webster).

The most extensive investigation on this subject in this country was probably that of Mitchell in Edinburgh, commenced in 1916 (published 1918).

(We will quote only results in children as these alone concern us). He examined tonsils from 2 groups of children.

(A) from those affected by Tuberculosis of the upper deep cervical glands. and

(B) from those with no clinical evidence of Tuberculosis of the cervical lymph nodes or other parts (i.e. cases of ordinary "enlarged Tonsils").

There were 100 children, therefore 100 pairs of tonsils in each group.

He examined the removed tonsils in 2 ways.

(1) by microscopic examination of sections of the Tonsils for evidence of Tuberculous pathological changes.

(2) by inoculation of guineapigs -- (the only method used by us).

By the inoculation method he found 21% of the tonsils positive for Tuberculosis, in Group A, and 9% positive in Group B. By the Microscopic method he found 38% positive in Group A. He explains the disparity in results of the two methods by the fact that many inoculated animals die of septic infection before tuberculous infection has time to develop.

It is noteworthy that no cases in either Group showed any evidence of Tuberculous infection of the tonsils naked eye -- either before or after removal. The age groups show that one half of the children in Group A were under 5 years of age, and one third of those in Group B were under 5 years.

Amongst Mitchell's conclusions we may note the following:

"(1) that primary tuberculosis of the faucial tonsils can be recognised only by the aid of the Microscope or by inoculation experiments.

"(2) that hypertrophied faucial tonsils are the seat of primary tuberculosis, though rarely as compared with tonsils from cases of tuberculous cervical adenitis.

"(3) that bovine and human types of tubercle bacilli are present in the tonsillar crypts of a small percentage of children without demonstrable tuberculous lesions either in tonsils or elsewhere".

We may therefore have tonsils containing tubercle bacilli, but showing no pathological changes whatever, (microscopically or naked eye); such cases can only be demonstrated by animal inoculation.

Mitchell also quotes 2 lists of interesting statistics given by other observers making similar investigations. Taking the average of these we find

(1) for Group A. (cases with tuberculous cervical glands) results of 10 observers give 40 positive out of 89 tonsils examined = 45%.

(2) for Group B. (ordinary cases of "enlarged tonsils" etc. with no signs of tuberculous adenitis, or tuberculosis elsewhere) results of 35 observers give 153 positive out of 4934 tonsils examined = 3.1%.

Mitchell also reports that milk samples examined in Edinburgh (1918)gave 20% positive for B. tuberculosis.

N.Wall (1922) examined Tonsils (and adenoids) from 120 patients by guineapig inoculation and found 3 positive (=2.5%) for B. tuberculosis.

Wilson and Nutt (1926) in Manchester found 13% of mixed milk samples contained B. tuberculosis.

Scarff and Whitby (1928) carried out in London investigations similar to those of Mitchell in Edinburgh. They used the same methods, but examined only tonsils from children who had enlarged tonsils but no tuberculous cervical adenitis. (Group B. of Mitchell).They examined 1st.series by histological method only, and 2nd.series by combined histological and animal inoculation methods--using 100 pairs of tonsils in each series.

They found the tubercle bacillus present in 2% by the histological method, and in 3% by the combined method.

They confirmed the observation of others that whilst tuberculous inflamation can usually be detected by histological examination, the mere harbouring of tubercle bacilli can only be detected by animal experiment.

These observers also report that the milk supply of London in 1926 gave 5% of samples positive for tubercle bacillus.

It is noteworthy that these positive findings are all much lower than those reported by Mitchell.

Scarff and Whitby conclude that this decrease in positive results is attributable to improved milk supply and education of the public regarding the danger of using infected milk.

These authors also quote the American observer, Weller, who examined (by histological method) tonsils from 8697 cases during the period 1906 to 1919. He found 2.35% positive for Bacillus tuberculosis. These included all age periods. In children 2--12 years of age he found 1.64% positive. (These cases were apparently not grouped into A and B.)

Vlasto (1931) discusses the question of Tonsils and Adenoids in relation to tuberculous infection. He concludes that primary tuberculosis of the tonsil is in most cases due to infection by the bovine bacillus (from ingestion of infected milk), and also that there is ample evidence that tuberculous adenitis of the neck has followed a primary invasion of the tonsil.

Webster (1932) examined tonsils from 46 children (2 to 14 years of age), who had simple hypertrophy, without adenitis or other signs of tuberculosis. None of them yielded the tubercle bacillus (animal inoculation method). On the other hand, in 86 cases with tuberculous cervical adenitis, he found 46.5% positive.

This author quotes several other investigations, which show an average of 10.13% positive for Group B., and 34.3% positive for Group A.(as grouped by Mitchell).

Comparing the above reports (1) in Group B, we notice a progressive decrease in the number of positives, Mitchell giving 9% (in 1918), Scarff and Whitby 2 to 3% (in 1928), and Webster - nil% (in 1932). This probably due (as suggested by Scarff and Whitby) to improved milk supplies (Tuberculin tested herds, Pasteurisation, etc.) and education of the public (many mothers boiling the milk, if not already safe-guarded).

(2) In Group A, we note the percentage of positives is persistently high. Mitchell gives 38% (1918) and Webster 46.5% (1932), and others vary from 34.3% to 45%. We would not expect these figures to decrease, however, owing to the nature of the cases. If the cervical glands have been <u>proved</u> to be tuberculous, the persistently high percentage of positive tonsils in such cases, confirms the hypothesis that the infection travels to the lymph nodes via the tonsils.

Scarff and Whitby (1928) also refer to the marked decrease in the number of patients requiring operative treatment for Tuberculous adenitis, e.g. at the Middlesex hospital the number of such in 1926 was half that in 1906 and less than 2/3 that in 1916.

They emphasise the necessity for Tonsillectomy in all cases where removal of Tuberculous glands in the neck is required. Without Tonsillectomy a focus of infection may remain active.

These agree with the conclusions of Mitchell

who also points out that primary Tuberculous infection of tonsils is attributable for the most part to drinking milk infected with the tubercle bacillus. Both bovine and human types of the bacillus may be present in tonsillar crypts; but most cases are bovine (Vlasto).

Part 11. The Present Investigation.

We inoculated altogether 115 guineapigs (including various controls etc.), with tonsil tissue, representing 100 pairs of tonsils. Of this series one guineapig showed typical tuberculous lesions in the spleen, liver, and inguinal lymph nodes. A smear from these last named was stained with Ziehl Nielsen and the presence of tubercle bacilli was established.

We have therefore 1% of our series of tonsils positive for Bacillus tuberculosis (inoculation method).

When collecting our tonsils notes were taken regarding clinical symptoms, previous illnesses, enlarged cervical glands, etc. in the children. From these we find that enlarged cervical glands were present in as many as 56% of the children of our series. Of these, however, only the tonsillar gland was affected (on one or both sides) in 21 cases (21%), as noted when the patients were examined prior to Tonsillectomy. If we deduct these, we have still 35% who either showed definite enlargement of the cervical glands at the time of examination (in the Out Patient Department), or who had a history of such glandular swelling. In

our one positive case for instance, the child, aged 3 years, had a history of a "lump on the right side of the neck lasting 14 days" and on examination the right tonsillar gland was definitely enlarged.

With such a large percentage giving evidence of cervical adenitis we would have expected a higher proportion of tonsils to yield the tubercle bacillus. We have no proof, however, that these were cases of tuberculous adenitis. There is no record of operation for such a condition in any of the series. We take it rather that the occurrence of cervical swelling (adenitis) had in most cases been comparatively recent, leading the parents to seek medical advice, and the doctor to recommend operative treatment (tonsillectomy); in the hope that the adenitis would subside and not recur after removal of the tonsils.

Our low percentage of positive tonsils would suggest that these cases of cervical swelling were in most instances non-tuberculous. They were probably due to the various pyogenic organisms which infect the tonsils, and which from these may pass on to the cervical lymphatic glands.

We may add that only in 2 or 3 of our cases were the cervical glands markedly enlarged, most of them being slight or moderate.

It is of interest to note that Morton (1933) reports finding the tubercle bacillus in 4.8% samples of pasteurised milk in Dundee, in 6.5% (samples of) raw milk, and in 8.5% retail milk.

Our results seem to correspond fairly closely to those of N.Wall (1922) and Scarff and Whitby (1928). We must also note that some of our animals were lost through septic infection, which these latter observers were apparently able to avoid.

Section IV (a). Conclusions.

(1) The tubercle bacillus was isolated from 1% of 100 pairs of tonsils examined by guineapig inoculation. These tonsils were from children suffering from "enlarged tonsils" etc.

(2) In 56% of these children, however, there was either a history or evidence of some cervical adenitis. In 21%, only the tonsillar gland was involved; and in the large majority of the others the swelling was slight of moderate. Our very low positive figure (1%) would suggest that these cases of cervical adenitis were probably mostly non-tuberculous.

(3) This low incidence of tuberculous tonsil infection is presumably attributable to general education of the public, better hygienic conditions, and improved milk supplies. Morton's statistics (1933) however (already quoted) suggest that there is room for improvement in this latter direction, including the pasteurised milk of Dundee, in which the incidence of B. tuberculosis is nearly 5%.

(b) The Occurrence of Micrococcus Flavus in Tonsils.

We have already referred to this group of organisms, -- Gram negative Flavoid cocci,-- in Section11. (p.126). When isolating Influenzoids by the Penicillin technique, these cocci were found to be very resistant to this reagent. We found that they formed a "scum" on the surface of the Penicillin broth cultures, and that their insensitiveness to Penicillin constituted the chief disadvantage of this highly successful and useful technique. We have already described the measures adopted to minimise this difficulty (p.128).

Our observations regarding these micrococci were quite superficial, but as we were meeting them constantly we made a few notes about them. (but only during the second half of the investigation).

1. Incidence in Tonsils.

They appeared to be almost universally present in the tonsils. In our subcultures from Penicillin Tonsil broths to Fildes Agar plates we found their pigmented colonies constantly present, intermingled with the colourless Influenzoids. They were present in about 90-95% of our tonsil series. When absent from these plates it was usually due to overgrowth with B. proteus.

The great majority of these plates showed

only colonies of Influenzoids and Flavoids, no other organisms being present; their relative numbers varied considerably. The technique used would have served admirably for isolation of Flavoids, as well as Influenzoids, had we been making a study of the former.

To illustrate the relative growths of these two groups of organisms on our Fildes agar plates we submit the following list of 14 consecutive cases.

	Flavoids	Influenzoids	Isolation of Influenzoid.
1.	++	+	not difficult
2.	++	+	" "
3.	+	+	" easy
4.	+	+ larger colonies	easy
5.	+	++ tiny colonies	"
6.	++	+	very difficult- replated.
7.	++	a few colonies	" " "
8.	++	nil	impossible "
9.	+	++	easy
10.	++	nil	impossible "
11.	+	++	easy
12.	+	++	"
13.	++	1 tiny colony	difficult
14.	++	" " "	"

The signs + ++ +++ indicate different degrees of growth.

These illustrate both the frequency of these cocci and the difficulty they cause in isolating

Influenzoids.

Others have recorded the frequent occurrence of these organisms in the throat and tonsils, e.g. N.Wall (1922) found them in 66% of over 100 tonsils.

2. Their Variability.

We noted this chiefly in regard to the different shades of colour -- yellow and brownish -- which they displayed. Some were very pale, others quite dark, and others again were brightly pigmented (yellow).

As mentioned before, a few strains resembled certain Influenzoid strains (brownish - but translucent) -- but were distinguished chiefly by their comparative opacity. (i.e. the colonies.).

There was no difficulty, however, in distinguishing at once the typical colourless Influenzoid colonies. S.P.Wilson (1928) and others refer to the great variations in the cultural characters of these organisms.

3. Suspension in Saline.

So far as tested these cocci were found to emulsify easily in water or saline. We did not, however, examine many strains in this way.

4. Resistance to Reagents.

We have already seen how insensitive these flavoid cocci are to Penicillin. In our experiments with Crystal Violet, however, we gathered that they are rather sensitive to this dye; they did not give

any trouble with this technique. (Section 1.) Fleming (1932) records that they are also sensitive to Tellurite, and that they differ in this respect from the pathogenic cocci (gonococcus, meningococcus, and M. catarrhalis). He suggests that this should form a useful means of differentiating between these pathogenic and non-pathogenic types of cocci which occur in the mouth and throat.

(c). Other Organisms encountered.

The only other organisms which we met with accidentally, and which seemed worth recording were C. diphtheriae and B. welchii. We simply record our findings.

C. diphtheriae was found in 2 instances; one was found to be gravis type, and one mitis (by Dr.Murray). This does not represent a percentage carrier-rate, as we did not look for this organism, but only met it unsought.

The only statistic we have at our disposal is that of Nakamura (1924) who found this organism in 3.6% of 841 tonsils examined.

B. welchii was found once only -- in a guineapig which died soon after inoculation. It showed a cellulitis of the abdominal wall with appearances suggestive of the presence of this type of organism. This was confirmed by microscopic examination.

We have not found any reference in the literature to the presence of this organism in tonsils.

Section IV. References.

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